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Salmonella spp. in swine - The abattoir as a link in the food chain

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Sê todo em cada coisa.
Põe quanto és no mínimo que fazes.

(*in* Odes de Ricardo Reis, Fernando Pessoa)

Aos meus Pais

Ao André, à Maria e à Francisca

DECLARATION/DECLARAÇÃO

In accordance with the provision of the Portuguese law nº 230/2009 of 13 October, the candidate state that was involved in the study design, execution of experimental work, in the analysis and interpretation of results, and in their preparation for publication, presented in this work.

No cumprimento do disposto no Decreto-lei nº 230/2009 de 13 de Outubro, como autora desta dissertação, declaro que participei na conceção dos estudos, execução experimental e interpretação dos resultados que estiveram na base do trabalho apresentado.

The work presented in this thesis was performed in the National Laboratory of Veterinary Investigation (Laboratório Nacional de Investigação Veterinária – LNIV), in the Microbiology Laboratory of the Faculty of Pharmacy of the University of Porto and the National Reference Laboratory of Antimicrobial Resistances, Department of Infectious Diseases, National Institute of Health Dr. Ricardo Jorge.

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Chapter 3

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Resumo

Salmonella é um dos agentes zoonóticos mais importantes a nível internacional, frequentemente implicado em doenças adquiridas por via alimentar. Os alimentos de origem animal são os seus principais veículos de transmissão ao Homem e, ao longo dos últimos anos, a carne de suíno tem sido identificada como uma fonte significativa de infeção humana. Assim, o objetivo deste trabalho foi caraterizar a ocorrência de *Salmonella* em suínos de abate, carcaças, carne e manipuladores de carne em matadouros portugueses, através da identificação dos processos de contaminação cruzada, bem como pela determinação dos perfis de resistência aos antimicrobianos nos isolados obtidos. O nível de conhecimento e prática dos manipuladores de carne foi também avaliado, de forma a clarificar a sua participação no processo de contaminação cruzada.

O presente estudo foi desenvolvido em oito matadouros e salas de corte e desossa no norte de Portugal. Foram avaliados 100 suínos abatidos, através da colheita de amostras de gânglios ileocecais, superfície externa da carcaça e carne de cada animal; as mãos dos manipuladores responsáveis pelas operações de corte e desossa de cada carcaça amostrada foram igualmente analisadas, através de zaragatoa. Assim, foi testado um total de 345 amostras (300 de suínos e 45 de manipuladores).

Na Secção III, Capítulos 1 e 2, os 60 isolados de *Salmonella* obtidos foram caraterizados fenotípica e genotipicamente, através da determinação do seu perfil de susceptibilidade aos antimicrobianos e identificação dos respectivos genes de resistência; a relação genética dos isolados foi avaliada através de eletroforese de gel em campo pulsado (PFGE). Os resultados obtidos revelaram uma elevada ocorrência de *Salmonella* em amostras de gânglios íleocecais (26,0%), seguida das carcaças (16,0%) e carne (14,0%). Contudo, os valores obtidos a nível ganglionar pareceram não demonstrar valor preditivo para resultados positivos nas amostras seguintes com a mesma proveniência. Por outro lado, os manipuladores foram identificados como uma possível fonte de contaminação subsequente, com 9,3% de resultados positivos.

No capítulo 1, foram identificados nove serótipos de *Salmonella enterica*, predominantemente *S.* Typhimurium (n=32) e a sua variante monofásica *S.* 4,[5],12:i:- (n=3), mas também *S.* Derby (n=11), *S.* Rissen (n=4), *S.* Mbandaka (n=3), *S.* London (n=3), *S.* Give (n=2), *S.* Enteritidis (n=1) e *S.* Sandiego (n=1), correspondendo a 17 perfis de PFGE, os quais se agruparam em 12 clusters e 5 perfis únicos. A resistência antimicrobiana foi identificada

em 75% dos isolados: tetraciclina (T, 70%), estreptomicina (S, 63%), sulfametoxazol (Sul, 62%), ampicillina (A, 57%) e cloranfenicol (C, 15%). Os fenótipos ASSuT (38%), ACSSuT (16%) e SSuT (13%) foram os mais frequentemente identificados, sendo que 63% dos isolados revelaram multirresistência. *S.* 4,[5],12:i:- foi identificada em suínos provenientes de 3 matadouros e surgiu associada a isolados de *S.* Typhimurium (cluster PFGE IT), com o fenótipo de resistência ASSuT, codificado pelos genes de resistência bla_{TEM-1}, *strA-strB, sul2* e *tet*(B). Contudo, um dos isolados de *S.* 4,[5],12:i:- apresentou o fenótipo de resistência ST, aqui pela primeira vez reportado, associado aos genes *strA-strB, tet(*A) e *tet*(B), dado que pode ser relevante, em termos do desenvolvimento que a variante monofásica emergente venha a assumir. *S.* Typhimurium DT104 (12%, cluster PFGE VIIT), isolada em amostras de gânglios ileocecais, carcaças, carne e um manipulador num único matadouro, demonstrando claramente o processo de contaminação cruzada, surgiu associada ao fenótipo ACSSuT e aos respectivos genes de resistência: bla_{PSE-1}, *floR, aadA2, sul1* e *tet*(A) ou *tet*(G). Os isolados de *S.* Rissen (cluster PFGE VIIIR), identificados num manipulador e em suínos provenientes de, respetivamente, 1 e 3 matadouros, apresentaram fenótipos de resistência distintos (T; AST; ASSulTW; ACSSulTW), associados aos genes *tet*(A), bla_{TEM-1}, *sul1* e/ou *sul3, aadA2, cmlA1* e *dfrA12*. *S.* London, identificada em 2 carcaças e um manipulador em 2 matadouros, é aqui reportada, pela primeira vez, com um amplo padrão de multirresistência (ANSSuT), associado aos genes bla_{TEM-1}, *strA-strB, sul2* e *tet*(A). A identificação de integrões de classe 1 (400-2000 bp) em 37% dos isolados e de genes de resistência específicos de clones internacionais isolados em humanos, já anteriormente detectados em Portugal, agora em suínos e manipuladores de carne, pode contribuir para compreensão da disseminação de clones de *Salmonella* multirresistentes, bem como da emergência da variante monofásica *S.* 4, [5],12:i:-.

Em suma, os resultados obtidos demonstram que, além dos elevados de valores de contaminação ao nível da produção suína, as operações do abate e de corte e desossa contribuem para a ocorrência e disseminação de clones relevantes em termos de saúde pública (*S.* Typhimurium DT104, a variante monofásica emergente *S.* 4,[5],12:i:- e *S.* Rissen) em produtos de origem suína. Este estudo permitiu também identificar uma possível via de contaminação da comunidade através dos funcionários dos matadouros.

Na segunda parte do estudo (Secção III, Capítulo 3), foi completado por manipuladores de carnes (n=159) um questionário de auto-aplicação desenhado para avaliar Conhecimento e Prática, associados a procedimentos de higiene pública. Uma percentagem significativa do grupo avaliado (72,7%) já tinha frequentado formação profissional, quer em Boas Práticas na Indústria Alimentar (12,03%), Higiene e Segurança no Trabalho (22,8%) ou em ambas as áreas (37,9%). Contudo, 24,5% dos manipuladores não tinham frequentado qualquer tipo de formação. Os manipuladores com formação em

Boas Práticas de Manipulação na Indústria Alimentar ou em ambas as áreas, obtiveram as proporções mais elevadas de respostas correctas, nos dois tipos de questões. Os resultados deste estudo apontam a necessidade de melhorar a formação, particularmente na área das Boas Práticas na Indústria Alimentar. O desenvolvimento de critérios de avaliação da eficácia da formação profissional é crucial para a protecção da saúde pública.

Em conclusão, os resultados apresentados nesta dissertação reforçam a necessidade de estratégias de intervenção no sentido de prevenir o desenvolvimento de *Salmonella* resistente aos antimicrobianos ao nível da produção suinícola, bem como a sua disseminação na cadeia alimentar através da contaminação cruzada nas operações do abate, com a participação dos manipuladores de carne.

Abstract

Salmonella is one of the most important zoonotic agents worldwide, frequently implicated in foodborne diseases. Food of animal origin is identified as being the main vehicle for transmission to humans. Over the last years, it has been recognized that contaminated pork is a significant source of human infections. Thus, the aim of the present work was to characterize the occurrence of *Salmonella* in slaughter swine carcasses, meat and meat handlers, in Portuguese abattoirs, tracking cross-contamination and antimicrobial drug resistance in the isolates. Furthermore, we targeted the evaluation of meat handlers' level of knowledge and practice, in order to clarify their participation throughout the cross-contamination process.

The present study was developed in eight abattoirs and deboning rooms of districts of Braga and Porto, in the north of Portugal. One hundred slaughtered pigs were sampled, collecting ileoceacal lymph nodes, a carcass external surface swab and meat samples from each pig. The hands of the meat handlers responsible for deboning operations in the sampled carcasses were swabbed. A total of 345 samples was analyzed (300 pig samples and 45 hand samples).

In Section III, Chapters 1 and 2 of results, 60 *Salmonella* isolates were phenotypically and genotypically characterized in order to access their antimicrobial resistance profiles and gain insight into the respective mechanisms of resistance; the genetic relatedness was established by pulsed-field gel electrophoresis (PFGE). The studies presented have shown a high frequency of *Salmonella* occurrence was found in the ileoceacal lymph node samples (26.0%), followed by carcass (16.0%) and meat samples (14.0%). However, ileoceacal lymph nodes that test positive for *Salmonella* are not found to be a predictor of positive test results further on in the process. Meat handlers were identified as a possible source of subsequent contamination, with 9.3% of the sample testing positive. Nine *Salmonella* enterica serotypes were detected, mainly *S.* Typhimurium (n=32) and the monophasic variant *S.* 4,[5],12:i:- (n=3), but also *S.* Derby (n=11), *S.* Rissen (n=4), *S.* Mbandaka (n=3), *S.* London (n=3), *S.* Give (n=2), *S.* Enteritidis (n=1) and *S.* Sandiego (n=1), belonged to 17 PFGE profile types corresponding to 12 clusters and 5 PFGE unique profiles. Antibiotic resistance was found in 75% of the clones, with 63% being multidrug-resistant (MDR). The highest resistance rates observed were to tetracycline (T, 70%), streptomycin (S, 63%), sulfamethoxazole (Sul, 62%), ampicillin (A, 57%) and chloramphenicol (C, 15%). The ASSuT (38%), ACSSuT (16%) and SSuT (13%) were the most frequent resistance phenotypes identified. *S.* 4,[5],12:i:- isolates,

which were recovered from swine in 3 abattoirs and clustered with *S.* Typhimurium isolates (PFGE cluster IT), were mostly associated to ASSuT phenotype, related with bla_{TEM-1}, *strA-strB, sul2* and *tet*(B) resistance genes. However, one of the *S.* 4,[5],12:i:- isolates presented the resistance phenotype ST here, firstly, reported associated to *strA-strB, tet*(A) and *tet*(B) genes, which may be relevant in the future development of the increasing importance of the monophasic variant. *S.* Typhimurium DT104 (12%, PFGE cluster VIIT) isolated in lymph nodes, carcasses, meat and a meat handler in 1 abattoir, clearly demonstrating cross-contamination, was associated with the ACSSuT phenotype, and bla_{PSE-1}, *floR, aadA2, sul1* and *tet*(G) or *tet*(A) genes. *S.* Rissen isolates (PFGE cluster VIIIR), recovered in swine and meat handlers of 3 and 1 abattoir, respectively, differed in the MDR profile (T; ASuTW; ASSuTW; ACSSuTW), associated to *tet*(A), bla_{TEM-1}, *sul1* and/or *sul3, aadA2, cmlA1* and *dfrA12* genes. Otherwise, *S.* London, identified in 2 carcasses and a meat handler in 2 abattoirs is here, firstly, reported with the ANSSuT MDR profile, associated to bla_{TEM-1}, *strA-strB, sul2* and *tet*(A) genes. Integrons (37% of isolates were positive for class 1 integrons, 400-2000 bp), and resistance genes of the main human clones spreading worldwide, including Portugal, were identified in swine and abattoir environment, which might contribute to the load of MDR *Salmonella* and to the emergence of monophasic variant S.4, [5],12:i:-.

Our results demonstrated that besides a high level of *Salmonella* swine contamination at the pre-harvest level, the slaughtering, cutting and deboning operations represents an important contribute to the occurrence of clinically relevant clones (e.g. *S.* Typhimurium DT104, the emergent *S.* 4,[5],12:i:- and *S.* Rissen) in pork products. This study also highlights the possibility of an ongoing MDR *Salmonella* community spread by abattoir workers.

In the third chapter of results, (Section III, Chapter 3), a self-administered questionnaire, designed to assess “Knowledge” and “Practice” of public hygiene measures, was completed by meat handlers (MH) (n=159). Seventy-three per cent of the group had professional training in two different areas: Good Practice in Food Industry (GPFI, 12.03%), Work Safety and Hygiene (WSH, 22.8%), and both (37.9%). However, 24.5% have no professional training. The results of this study point to the need to improve training, particularly in Good Practice in Food Industry, since meat handlers with professional training in GPFI and in both areas had the highest proportions of correct answers. The development of evaluation criteria for the effectiveness of professional training is crucial to protect public health.

Globally, the presented results reinforce the need of intervention strategies, preventing MDR *Salmonella* development in the pre-harvest stage, as well as the spreading in the food chain, through slaughter operations and meat handlers’ participation.

Abbreviations

attI – adjacent recombination site
B cells – Bone marrow dependent lynfocytes
CDC – Centers for Disease Control and Prevention
cfu – colony forming units
DNA – Deoxyribonucleic acid
DT – Definitive phage type
ECDC – European Centre for Disease Prevention and Control
EFSA – European Food Safety Authority
EU – European Union
GALT – gut-associated lymphoid tissue
GMP – Good manufacturing practice
HACCP – Hazard Analysis Critical Control Points
ICBAS – Instituto de Ciências Biomédicas Abel Salazar
int – integrase gene
IR – Inverted-repeated sequences
IS – Insertion sequence
ISCR – Insertion sequence common region
MGE – Mobile genetic elements
MS – Member states
Pc – promoter
PCR – Polymerase chain reaction
PFGE – Pulsed-field gel electrophoresis
PRRSV – Porcine reproductive and respiratory syndrome virus
R type – Resistance type
T cells – Thimus dependent lynfocytes
u.v. – Ultraviolet
USA – United States of America
USDA – United States Department of Agriculture
WHO – World Health Organization

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SECTION I

INTRODUCTION / LITERATURE REVIEW

1. Introduction

Salmonella is a zoonotic pathogen able of colonizing many vertebrate hosts. Infections due to *Salmonella* in humans and domestic animals are important worldwide problems and the results diverge from asymptomatic carrier stages to severe systemic infections. Non-typhoidal salmonellosis is regarded as one of the most important foodborne zoonotic diseases being *Salmonella* the most frequent etiologic bacterial agent of foodborne disease outbreaks, causing ill health and high disease-related costs in the human society (De Jong Skierus, 2006; European Food Safety Authority, 2012a, 2012b; Mølbak, Olsen, & Wegener, 2006; Newell et al., 2010). The economic impact of this zoonosis in commercial food production is also substantial and control of *Salmonella* is becoming more challenging with the trend towards cheaper and faster food (Fullerton, 2008; Hendriksen et al., 2011; Rhen, Maskell, Mastroeni, & Threlfall, 2007). *Salmonella* infections in animals are important due to the economic consequences of the morbidity and the mortality, related with clinical disease and to human health consequences of disease, developed by direct or indirect contact with animals or animal products (Denagamage, 2008). *Salmonella* has long been recognized as an important zoonotic pathogen particularly transmitted through food or food chain, causing a wide spectrum of diseases such as acute gastroenteritis, bacteremia, and extra-intestinally localized infections involving many organs (Su, Chiu, Chu, & Ou, 2004). In humans, non-typhoidal salmonellosis is typically characterized by an acute gastrointestinal illness, with symptoms such as fever, diarrhea, abdominal pain, nausea and occasionally vomiting. The symptoms normally appear within 12-72 hours after infection. Those most severely affected by *Salmonella* are individuals with a less effective immune system, such as young, old, pregnant and immunocompromised persons (Boyen et al., 2008). Those patients are also more disposed to develop bacteremia and sometimes life-threatening extra-intestinal infections such as meningitis, osteomyelitis, septic arthritis, cholangitis and pneumonia (Acheson & Hohmann, 2001; Hsu, Tsay, Chen, & Chu, 2003).

In the United States of America (USA) the total annual number of human cases of non-typhoidal salmonellosis has been estimated to be approximately 1.4 million, annually resulting in more than 500 deaths (Scallan et al., 2011; Voetsch et al., 2004). Within the European Union (EU), *Salmonella* spp. was the second most frequently reported microorganism causing zoonotic disease in humans in 2010, with 99 020 confirmed cases of salmonellosis reported, giving 21,5 cases per 100 000 population (European Food

Safety Authority, 2012a). The World Health Organization (WHO) reports that the incidence and severity of cases of salmonellosis have increased significantly (World Health Organization, 2009).

Salmonella infections are commonly self-limiting and the treatment with antibiotics is therefore, most often not required (Anjum et al., 2011; Casburn-Jones & Farthing, 2004). Nevertheless, under specific clinical circumstances, approximately 5% of individuals with gastrointestinal illness caused by non-typhoidal *Salmonella* will develop bacteremia, a serious and potentially fatal problem that requires antibiotic treatment (Alcaine, Warnick, & Wiedmann, 2007; Anjum et al., 2011; Su et al., 2004). This treatment has been successful in the past but new multi-drug-resistant (MDR) *Salmonella* strains are rapidly emerging since the 1990s and constitute a serious additional concern for public health (Boyen et al., 2008; European Food Safety Authority, 2012a; Mastroeni, Chabalgoity, Dunstan, Maskell, & Dougan, 2001; Newell et al., 2010; World Health Organization, 2009).

2. *Salmonella* – A food-borne zoonosis of increasing importance

In the last decades, it was verified an increasing interest on *Salmonella* spp. as a foodborne zoonosis agent and the relevance of it in a public health approach. This literature review subsequently presented, does not pretend to be a whole revision in all domains. The effort was focused on the main aspects interesting for our work: the link between slaughter swine *Salmonella*-carriers and the presence of the agent in meat and meat handlers, with their implications on transmission dynamics and public health.

2.1. Historical aspects

Salmonellae were first observed by Eberth in lymphatic tissue from a human patient who died from typhoid fever in 1880 (Mastroeni & Maskell, 2006). The organism we today know as *Salmonella* Cholerasuis was identified in 1885 in the United States Department of Agriculture (USDA), by the pathologist and microbiologist Theobald Smith in a strain isolated from pigs suffering of swine fever (Chernin, 1987). It was named after Dr. Daniel Elmer Salmon, his hierarchic superior in the USDA, a Veterinary Surgeon responsible for a number of significant public health policies, including a nationwide system for meat inspection and quarantine requirement for imported livestock. During the study of hog cholera, together they also demonstrated that dead organisms could immunize animals against living organisms. This was the foundation to the development of a vaccine against typhus (Chernin, 1987; Hoogstral, 1986; Wray & Wray, 2000).

The first report of an outbreak of foodborne salmonellosis is from 1888 and described an episode in Germany in which 58 persons who had eaten beef developed acute gastroenteritis; one of them died (Mølbak et al., 2006). In the following years, a number of outbreaks of salmonellosis affecting man or animals were reported and the notion of "meat poisoning" was associated to the etiologic agent *Salmonella*. Human salmonellosis occurred predominantly among individuals who ate meat from ill animals, mostly cattle, pigs and goats (Mølbak et al., 2006). An important episode occurred in Alvesta, Sweden, in 1955, when a meatborne outbreak of *S.* Typhimurium affected 9000 people causing 10% of deaths and provoking an early implementation of *Salmonella* control in Sweden (Bengtsson, Hedlund, Nisell, & Nordenstam, 1955). In several European countries, there are reports of rodenticides using cultures of *S.* Enteritidis and human disease associated to rat baits handling or accidental ingestion (Painter et al., 2004). While *S.* Typhi became a vast problem in the early industrial era, particularly in the USA, the burden associated with non-typhoid *Salmonella* was low before World War II (Tauxe, 1998). Advances in sanitation practically eliminated *S.* Typhi as a cause of indigenous infections in developed countries. Many decades later, non-typhoid *Salmonella* infections began to rise in importance, a tendency that may have reached the maximum in the past two decades (Mølbak et al., 2006). On the consequence of that, most developed countries have been now laboratory-based surveillance of *Salmonella* infections, including notification and recording outbreaks systems.

2.2. Characteristics, taxonomy and nomenclature of *Salmonella*

Salmonella is a typical member of the family *Enterobacteriaceae* and consists of non spore-forming Gram-negative bacilli. Within *Enterobacteriaceae*, *Salmonella* has its closest relatives in *Escherichia coli* and *Shigella*. *E. coli* and *Salmonella* are thought to have evolved from a common ancestor 140 million years ago (Wray & Wray, 2000). Most *Salmonella* isolates grow optimally at 35-37°C, with a most adequate pH=6,5-7,5; however, they can also grow between 7°C and 48° C, tolerating pH between 4 to 8 (Delhalle, Saegerman, Messens, et al., 2009). Under optimal conditions generation time is 25 minutes, but the growth in food is generally inhibited in the presence of 4% of NaCl (Delhalle, Saegerman, Messens, et al., 2009). Most *Salmonella* serotypes are readily killed by heat (i.e. cooking to a core temperature of 70°C for 2 minutes), but can survive freezing and drying (Delhalle, Saegerman, Messens, et al., 2009; Denagamage, 2008). Bacteria constituting the genus contain three different types of antigens. The agglutinating properties and antigenic polymorphisms of the somatic O, flagellar H and capsular Vi are used to differentiate between more than 2 500 serologically distinct types (serotypes or serovars) of *Salmonella* (Grimont & Weill, 2007; Popoff, Bockemuhl, & Gheesling, 2003;

Velge, Cloeckaert, & Barrow, 2005). Although broad serotyping of all surface antigens can be used for formal identification, most clinical microbiological laboratories accomplish a few simple agglutination reactions to define specific O antigens into serogroups, designated groups A, B, C1, C2, D, and E (Farmer, 1995). The development of serotyping was fundamental for the understanding of the epidemiology of *Salmonella* infections (Mølbak et al., 2006). This grouping system can be used clinically to confirm genus identification; however, it cannot quickly identify whether the organism is likely to cause enteric fever, because considerable cross-reactivity among serogroups occurs (Chiu, Su, & Chu, 2004).

The genus *Salmonella* is currently divided into two species: *S. enterica* and *S. bongori*, each of which contains multiple serotypes. *S. enterica* is further divided into six sub-species: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI) (Popoff & Le Minor, 1997). The vast majority (99.5%) of strains of *Salmonella* isolated from humans and warm-blooded animals belong to sub-species I (Grimont & Weill, 2007), while the other five sub-species II-VI and *S. bongori* are primarily associated with cold-blooded animals and are only infrequently isolated from mammals (Foti et al., 2009; Nastasi, Mammina, & Salsa, 1999). *S. enterica* sub-species *enterica* contains all serovars able to promote illness in domestic animals and humans and only less than fifty serovars are responsible for most of the cases of disease (Denagamage, 2008). Members of this sub-species usually are named based on where the serovar or serotype was first isolated. For named serotypes, to emphasize that they are not separate species, the serotype name is not italicized, and the first letter is capitalized. At the first citation of a serotype, the genus name is given followed by the word “serotype” or the abbreviation “ser.” and then the serotype name (for example, *Salmonella* serotype or ser. Typhimurium). Subsequently, the name may be written with the genus followed directly by the serotype name (for example, *Salmonella* Typhimurium or *S.* Typhimurium) (Brenner, Villar, Angulo, Tauxe, & Swaminathan, 2000). Serotypes belonging to another sub-species are designated by their antigenic formulae, following the sub-species name (Popoff, Bockemuhl, & Gheesling, 2004). The simplified antigenic formulae of *Salmonella* serovars are listed in a document called the Kauffmann-White scheme (Popoff et al., 2003). The antigenic formulae of *Salmonella* serotypes are defined and maintained by the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France, and new serotypes are listed in annual updates of the Kauffmann-White scheme.

Strains defined as *S.* Typhimurium possess two phases of H-antigens: in phase 1 this is H-antigen “I” and in phase 2 they are H-antigens “1, 2”. These are universally regarded as “classic” *S.* Typhimurium strains (antigenic formula: 1,4,[5],12:i:1,2). Antigenic variants that lack either the first or the second phase H antigen, or both, have been described (antigenic formulas respectively: 1,4,[5],12:-:1,2, or 1,4,[5],12:i:-, or 1,4,[5],12:-:-). Such variants have

been termed "*Salmonella* Typhimurium-like" strains. Within these *Salmonella* Typhimurium-like strains, monophasic variants lacking the second phase H antigen (1,4,[5],12:i:-) are referred to as "monophasic *S.* Typhimurium" (European Food Safety Authority, 2010b).

A second level of characterization is based on phage typing. By use of 37 different phages, serotype Typhimurium can be divided into more than 210 phage types (Anderson, Ward, Saxe, & De Sa, 1977; Botteldoorn, Herman, Rijpens, & Heyndrickx, 2004). Besides serotyping and phage typing, bacterial molecular typing methods, such as plasmid profiling, pulsed-field gel electrophoresis (PFGE), IS200 typing, ribotyping, random amplified polymorphic DNA analysis, and amplified fragment length polymorphism, are used for epidemiological investigation of salmonellae (Baggesen, Sandvang, & Aarestrup, 2000; Daly & Fanning, 2000; Ebner & Mathew, 2001; Liebana, Garcia-Migura, Breslin, Davies, & Woodward, 2001; Liebana et al., 2002; Olsen, Skov, Angen, Threlfall, & Bisgaard, 1997). These techniques are useful for describing clonal relationships between strains (On & Baggesen, 1997) and for assessing the distribution of *Salmonella* strains within food-processing environments (Botteldoorn et al., 2004; Giovannacci et al., 2001; Millemann, Lesage, Chaslus-Dancla, & Lafont, 1995). The use of polymerase chain reaction (PCR) assays for the identification of specific serotypes, as *Salmonella enterica* serotype Typhimurium DT 104 and U302 (Pritchett, Konkel, Gay, & Besser, 2000) and *Salmonella enterica* 4,[5],12:i:- (Soyer et al., 2009; Tennant et al., 2010) is also available.

2.3. Hosts and habitats

Salmonellae can be frequently found in sewage, sea, and river water and can contaminate a variety of foods. The microorganisms have been isolated from many animal species including, cows, pigs, chickens, turkeys, pigeons, sheep, dogs, cats, horses, donkeys, seals, lizards and snakes (Foti et al., 2009; Grimont & Weill, 2007; Haraga, Ohlson, & Miller, 2008; Mandell, Douglas Jr, & Bennett, 1979; Mølbak et al., 2006). Furthermore, migratory birds, amphibians, fish and even insects can also be infected by *Salmonella* spp. (Foti et al., 2009; Greenberg, Kowalski, & Klowden, 1970; Mitscherlich & Marth, 1984; Wells, Boulton, Hall, & Bidol, 2004). *Salmonella* is generally regarded as part of the normal intestinal flora of reptiles kept as pets (Österberg, 2010; Warwick, Lambiris, Westwood, & Steedman, 2001), suggesting that wild terrestrial reptiles may work as reservoirs (Briones et al., 2004; Hidalgo-Vila, Díaz-Paniagua, de Frutos-Escobar, Jiménez-Martínez, & Pérez-Santigosa, 2007). Some *Salmonella* species are restricted to one or few animal species, whilst others have a wider host spectrum (Mastroeni et al., 2001). According to the European Food Safety Authority (EFSA), all serotypes of *Salmonella enterica* are potentially hazardous to human health and thus regarded as pathogens (European Food Safety Authority, 2010b). Nevertheless, the majority of *Salmonella*

infections reported in humans, and domestic animals are caused by relatively few of the more than 2500 identified serotypes (Hendriksen et al., 2011; Österberg, 2010). Although most of the serotypes of *Salmonella enterica* sub-species *enterica* have the capability to colonize the alimentary tract of a wide range of animals, a few have a predilection for one or a few host species (Österberg, 2010). The serotypes may be divided into three groups: 1. host-specific serotypes, 2. host-restricted serotypes and 3. broad host range serotypes (Mastroeni & Maskell, 2006; Uzzau et al., 2000; Uzzau et al., 2001) (Table 1). The typhoid salmonellae (*S.* Typhi and *S.* Paratyphi A, B, and C) and *S.* Sendai remains important and exclusive pathogens in humans in developing countries and are able to cause a severe, systemic disease referred to as 'enteric fever' (Giaccone, Catellani, & Alberghini, 2012; Mølbak et al., 2006). Typhoid fever in humans is still endemic in many developing countries in Africa and Asia often owing to fecal contamination of water supply, affecting approximately 21 million individuals annually, with a mortality of 1% (Crump et al., 2003). Prevention of the disease by implementation of hygiene measures is possible, but can be difficult (Mastroeni et al., 2001).

Table 1. Examples of *Salmonella* serotypes and their host-specifity (from Österberg, 2010)

Group	***Serotype***	***Main host***	***Other host***
Host specific	*S* Typhi,	Human	
	S Paratyphi	Human	
	S Abortusovis	Sheep	
	S Gallinarum	Poultry	
	S Abortusequi	Horse	
Host restricted	*S* Cholerasuis	Swine	Human
	S Dublin	Cattle	Human
Broad host range (ubiquitous)	*S* Typhimurium		
	S Enteritidis		

The ability to survive outside the host is considered an essential piece of the epidemiology of *Salmonella* spp. In stored samples of feed, grass or dust, spiked with 106-108 colony-forming units (cfu) of *S.* Typhimurium per gram, survival times of one year are not uncommon and up to four years have been reported (Mitscherlich & Marth, 1984). In addition, in liquid manure, *S.* Typhimurium was re-isolated after 140 days at +10ºC (Gudding, 1975). In field experiments, the survival times have not been rather than long, but still at least weeks to months dependent on temperature and humidity (Holley & Guan, 2003; Semenov, Van Overbeek, & Van Bruggen, 2009). The feature of being able to survive and sometimes even replicate in varying environments promotes the ubiquitous

presence of *Salmonella* spp. and complicates its control. An element assumed to be essential for the persistence in the environment, as well as for the colonization in the host, is the biofilm formation defined as 'bacterial communities enclosed in a self-producing matrix adherent to each other and/or surfaces or interfaces' (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995; Costerton, Stewart, & Greenberg, 1999). This multicellular structure allows the bacteria to adapt to divergent surfaces ranging from the epithelial cell layer in the intestine to the stainless steel in feed factories, meat plants or animal transport trucks. It is suggested that biofilm formation facilitate the persistence by protecting bacteria against environmental stress such as disinfection and desiccation (Vestby, Møretrø, Langsrud, Heir, & Nesse, 2009). These different habitats provide opportunities for adaptation and evolution, and this is revealed by the changing trends in salmonellosis observed in recent years (Newell et al., 2010). A further trend recently identified in *Salmonella* infections has been an increased association of outbreaks with previously unusual vehicles, like fresh produce. Many such harvests are produced in developing countries where manure is regularly used as a natural fertilizer. Recent studies suggest that some *Salmonella* spp. have now evolved to attach to and colonize vegetables (Barak, Gorski, Naraghi-Arani, & Charkowski, 2005; Franz & Van Bruggen, 2008; Islam et al., 2004; Klerks, Franz, van Gent-Pelzer, Zijlstra, & Van Bruggen, 2007). Thus, it seems that *Salmonella* spp. are remarkably adaptable organisms able to evolve to fill different niches and respond to environmental challenges, improving survival mechanisms and providing new host and novel habitat opportunities (Newell et al., 2010).

2.4. Transmission pathways

Salmonellae may be transmitted through direct contact with infected animals or between humans, or from environments contaminated with feces. Transmission also occurs when organisms are introduced in food preparation areas and are allowed to multiply in food, due to inadequate storage temperatures, insufficient cooking, improper handling and cross contamination of ready-to-eat food (European Food Safety Authority, 2011c). Humans can be healthy carriers of *S. enterica* in the intestine. This may be a potential hazard to food hygiene, if the carriers are the people involved in producing and handling the food. Usually an asymptomatic carrier eliminates *Salmonella* in their feces for several months after the episode of gastroenteritis through which they became a carrier (Giaccone et al., 2012). Food-borne outbreaks of salmonellosis are consistently observed and regularly reported. This is a reflection of a low infectious dose in humans, an ability to grow in unprocessed food and in the environment allowing amplification, and long-term survival and, therefore, ease of recovery from contaminated foods (Giaccone et al., 2012; Newell et al., 2010).

Although *Salmonella* spp. may survive for long periods in the environment, it is believed that the carrier animal is the major source of infection for both animals and humans (Fedorka-Cray, Gray, & Wray, 2000). The common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals, which results in a variety of foodstuffs, of both food of animal and plant origin, as sources of human infections. From the intestinal contents of livestock, the salmonellae can contaminate fresh meat, raw milk and egg shells (Giaccone et al., 2012). If the necessary hygienic precautions are not taken in the early stages of the production line (slaughter, milking, egg collecting), there is a risk that the salmonellae may then extent along the food chain, contaminating products such as cured meats, dairy and egg-based products, the main cause of salmonellosis in developed countries (Giaccone et al., 2012). Furthermore, through the feces of animals and man, salmonellae can contaminate farmland, surface water flow and vegetables if they are fertilized with animal manure or manure that is not properly fermented. Vegetables, therefore, can be a source of disease to humans just like fresh meat, milk, shell eggs and by-products (Franz & Van Bruggen, 2008; Klerks et al., 2007). Besides in animals, *Salmonella* can adhere well to the work surfaces, and from there extent to other foodstuffs by cross-contamination (Møretrø, Heir, Nesse, Vestby, & Langsrud, 2012). Additionally, contaminated animal feed may constitute a source of infection with *Salmonella* spp. in animals (Crump, Griffin, & Angulo, 2002; Davies, Scott Hurd, Funk, Fedorka-Cray, & Jones, 2004; Österberg, 2010). *Salmonella* in feed may derive from contaminated ingredients or from environmental contamination of the feed during crushing or subsequent feed production processes (Binter et al., 2011; O'Connor, Denagamage, Sargeant, Rajić, & McKean, 2008; Österberg, 2010). In recent EU data, it was detected from 0% to 3.6% in pig feed samples (European Food Safety Authority, 2012a).

3. *Salmonella* in swine and pork as a foodborne pathogen

Food-producing animals, particularly poultry and swine, are considered to be the primary reservoir of non-typhoidal *Salmonella*, causing enteric infection in humans (Carattoli, 2008). Swine are frequently asymptomatic *Salmonella* carriers who play a main role as a primary source of contamination of the environment, other animals and fresh or processed meat (Gopinath, Carden, & Monack, 2012). Pork is an important source of *Salmonella* infections for humans, mostly related with *S.* Typhimurium (Boyen et al., 2008; Buchholz et al., 2005; Gebreyes, 2008; Gebreyes, Thakur, Davies, Funk, & Altier, 2004; Mølbak et al., 1999; Thakur, Tadesse, Morrow, & Gebreyes, 2007; Threlfall, 2000) and pork-related outbreaks with a fatal outcome have been described (Jansen, Frank, & Stark, 2007).

Indeed, in USA, statistical models have predicted that every year nearly 100,000 human cases of salmonellosis are related to the consumption of pork, with a resultant annual social cost of approximately 80 million dollar (Miller, Liu, McNamara, & Barber, 2005). In the EU, it is estimated that around 28% of the human salmonellosis cases are attributable to pigs and pork (European Food Safety Authority, 2011b).

The *Salmonella* situation at the farm-level has recently started to become an issue in various countries, coinciding with growing concern regarding food safety and problems associated to large-scale industrial pork production (Crump et al., 2002; Davies, 1997; Davies et al., 2004; Fraser, 2006; Kich et al., 2007; Kich et al., 2011; Molla et al., 2010). Stress factors, such as feed withdrawal from swine prior to slaughter and transport to the abattoir, have been shown to promote shedding of *Salmonella* by carrier swine (Berends, Urlings, Snijders, & Van Knapen, 1996; Berends, Van Knapen, Mossel, Burt, & Snijders, 1998a, 1998b; Botteldoorn et al., 2004; Boyen et al., 2008; De Busser et al., 2011; Delhalle, Saegerman, Farnir, et al., 2009; Lo Fo Wong, Hald, van der Wolf, & Swanenburg, 2002; Lo Fo Wong et al., 2003; O'Connor et al., 2008; Swanenburg, Urlings, Snijders, Keuzenkamp, & Van Knapen, 2001; Swanenburg, Van der Wolf, Urlings, Snijders, & Van Knapen, 2001). This release of *Salmonella* contributes to the contamination of carcasses and the environment at the slaughterhouse, threatening meat consumers (De Busser et al., 2011; Delhalle, Saegerman, Farnir, et al., 2009; Delhalle, Saegerman, Messens, et al., 2009; Hald, Lo Fo Wong, & Aarestrup, 2007; Letellier, Messier, Paré, Ménard, & Quessy, 1999; Lo Fo Wong et al., 2003; Vieira-Pinto, Temudo, & Martins, 2005; Vieira-Pinto, Tenreiro, & Martins, 2006).

The public health risk of *Salmonella* infection from ingestion of contaminated pork relies on multiple factors. Including the level of infection in the pig herd (European Food Safety Authority, 2011c; Hill et al., 2003; Nollet et al., 2005), hygiene during carcass processing in the abattoir (Berends et al., 1998a; Borch, Nesbakken, & Christensen, 1996; De Busser et al., 2011; Delhalle, Saegerman, Messens, et al., 2009; Swanenburg, Urlings, et al., 2001; Swanenburg, Van der Wolf, et al., 2001), meat storage and distribution conditions (Delhalle, Saegerman, Farnir, et al., 2009; Mann, Smith, & Brashears, 2004) and finally, the handling of undercooked pork by the consumer (Hill et al., 2003).

3.1. Infection and pathogenesis

Swine usually get infected by oral route and can carry *Salmonella* asymptomatically in the tonsils, the intestines and the gut-associated lymphoid tissue (GALT) (Boyen et al., 2008; Fedorka-Cray et al., 2000; Mastroeni & Maskell, 2006; Scherer et al., 2008; Wood, Pospischil, & Rose, 1989). The transmission of the infection is facilitated by low hygiene standards and/or dense populations facilitating faecal contamination of feed or

the environment (Österberg, 2010). Except for infections with *Salmonella* Typhisuis, Cholerasuis and some types of *S.* Typhimurium, *Salmonella* infections of pigs are practically always subclinical (Boyen et al., 2008; Huang, Lin, & Wu, 2009).

Infections with the host-adapted serotype Choleraesuis often occur in North America and Asia being only occasionally described in Western Europe or Australia (Chang et al., 2005; Chiu et al., 2004; Fedorka-Cray et al., 2000; Nollet et al., 2006). Disease associated with this serotype is characterized by septicemia, enterocolitis or bacteremia localization as pneumonia and hepatitis or sporadically as meningitis, encephalitis and abortion (Boyen et al., 2008; Haesebrouck et al., 2004; Huang et al., 2009). In Western Europe, *S.* Typhimurium is responsible for most of the cases of clinical salmonellosis in swine (Boyen et al., 2008; Haesebrouck et al., 2004). This serotype is mainly related with enterocolitis including a febrile phase with dullness and loss of appetite, watery diarrhea and reduced general condition, followed by recovery with continued excretion of the bacteria for varying time periods (Boyen et al., 2008; Griffith, 2006). Although these infections may result in enteric and fatal systemic disease, this serotype frequently passes sub clinically in swine (Boyen et al., 2008; Haesebrouck et al., 2004). After infection with *S.* Typhimurium, pigs may develop a carrier state, excreting intermittently the bacteria for up to 28 weeks, in spite of a declining after the first 2 weeks, without presenting clinical signs (Haesebrouck et al., 2004; Scherer et al., 2008; Wood et al., 1989). Single oral doses of among 101-103 cfu *Salmonella* spp. can be sufficient to infect about 0.1 to 10% of exposed animals. Additionally, as little as 2 cfu g^{-1} *Salmonella* spp. of feed may be sufficient to infect farm animals (Wray, Todd, McLaren, Beedell, & Rowe, 1990). After oral or aerogenic uptake of *Salmonella* bacteria, the tonsils and distal intestinal tract (ileum, caecum and colon) are colonized (Boyen et al., 2008; Fedorka-Cray, Kelley, Stabel, Gray, & Laufer, 1995; Marg, Scholz, Arnold, Rösler, & Hensel, 2001; Wood et al., 1989). The palatine tonsils are frequently severely infected and should, therefore, not be underestimated as a source of *Salmonella* contamination during slaughter (Kühnel & Blaha, 2004; Vieira-Pinto et al., 2005; Wood et al., 1989). The infection occurs in common in three different steps, after oral inoculation: (a) colonization of the gut and adhesion to the wall, (b) invasion of the wall, (c) dissemination to mesenteric lymph nodes and other organs (Berends et al., 1996; Boyen et al., 2008; Scherer et al., 2008). Underneath definite conditions, such as during transport, infections may also occur directly via the tonsils, whereby the agent, within 2-6 h, can reach the colon and rectum via lymphatic routes (Berends et al., 1996; Reed, Olander, & Thacker, 1986). Colonization of the gut happens when enough numbers are ingested to pass through the stomach, or after multiplying in the oropharynx and tonsils (Wood et al., 1989). The acid environment of the stomach establishes an obstacle and diminishes the number of viable *Salmonella* bacteria (Giannella, Broitman, & Zamcheck,

1972; Haesebrouck et al., 2004). Every situation upsetting the pH of the stomach thus improves the number of salmonellae that reach the small intestine and stimulate effective colonization. In the proximal part of the small intestine, bile inhibits invasion of the mucosa by suppressing the *Salmonella* intestinal invasion mechanism (Galán, 2001; Haesebrouck et al., 2004; Prouty & Gunn, 2000), this might explain why *Salmonella* preferentially colonizes the ileum, caecum and colon (Boyen et al., 2008). In the intestinal wall, salmonellae are located in and between the enterocytes and in macrophages and leucocytes, but because bacteria can survive and proliferate in macrophages and leucocytes, translocation to lymph nodes and other organs will certainly happen (Wells, Maddaus, & Simmons, 1988). Neutrophils are attracted to the intestinal lamina propria and migrate towards the lumen. This process is related with the development of diarrhea (Boyen et al., 2008). The neutrophils in the gut belong to the first line of defense against a *Salmonella* infection, hence inefficient uptake by them may provide an opportunity for the pathogen to colonize and/or replicate to levels that enable development of a carrier state or clinical infection in pigs (Stabel, Fedorka-Cray, & Gray, 2002). The presence of high numbers of neutrophils in the porcine gut allows the host to overcome a *Salmonella* infection (Foster et al., 2003; Foster, Hulme, Lovell, Reed, & Barrow, 2005). Conversely, the damage induced by activated neutrophils is considered the main cause of the gut pathology distinctive for *Salmonella* infections (Boyen et al., 2008; Tükel et al., 2006).

When the *Salmonella* bacteria have reached the intestinal lamina propria again they stimulate their uptake, this time by macrophages. Inside these macrophages, *Salmonella* is capable of surviving and even multiplying (Haesebrouck et al., 2004; Hensel, 2000). The safe position, which the macrophage offers, permits the bacteria to extent intra-cellularly all through the body and reaches the internal organs (Haesebrouck et al., 2004). Eventually, *Salmonella* induces an apoptosis-like process in the host macrophage (Van der Velden, Lindgren, Worley, & Heffron, 2000). This process permits the uptake of bacteria by other macrophages and consequently, stimulates bacterial dispersion (Haesebrouck et al., 2004). The macrophage death promotes fast dispersal in macrophages of the intestinal mucosa and spreading in the internal organs (Boyen et al., 2008). In systemic infections, the bacteria reach the phagocytes of the spleen, liver and bone marrow (Mastroeni et al., 2001), conversely there is no detection at these organs in *Salmonella*-carrier pigs (Scherer et al., 2008). Complement activation at the bacterial surface or the presence of opsonizing serum antibodies facilitates the uptake of the organisms by phagocytes (Liang-Takasaki, Saxen, Makela, & Leive, 1983; Mastroeni et al., 2001; Mastroeni & Maskell, 2006; Saxén, Reima, & Mäkelä, 1987). During systemic infections, the majority of salmonellae are associated with macrophages and polymorphonuclear phagocytes and the ability to grow within these cells seems to be a requirement for *Salmonella* virulence (Mastroeni et al.,

2001). When high bacterial numbers are extended in the tissues, salmonellae can be seen also in the extracellular compartment and in non-phagocytic cells, namely hepatocytes. (Conlan & North, 1992; Hsu, 1989; Mastroeni et al., 2001).

Studies showed that pigs which were orally administered 10^9 cfu of several types of *Salmonella* spp. excreted the organisms within 24 h. In slaughter swine, at the moment the first animals were slaughtered (8 h after oral inoculation) their mesenteric lymph nodes were already positive (Berends et al., 1996; Wood et al., 1989). Furthermore, experiments with weaned pigs demonstrated that lymphoid tissues closely associated with the digestive tract, such as the tonsils and the mesenteric lymph nodes, may harbor *Salmonella* spp. for 28 weeks or longer, but that other lymph nodes, such as the axillary or inguinal lymph nodes, only contain them for a period of 2-4 weeks (Berends et al., 1996; Wood et al., 1989). Regarding the spread of infections between pigs, excretion in the feces is particularly important (Berends et al., 1996; Boyen et al., 2008; Scherer et al., 2008).

3.2. Vaccination and immunity

In swine, the role of the immune-status of individuals concerning *Salmonella* is not completely clear. Host resistance to *Salmonella* relies initially on the production of inflammatory cytokines leading to the infiltration of activated inflammatory cells in the tissues (Mastroeni et al., 2001; Mastroeni & Ménager, 2003; Mastroeni & Maskell, 2006). Thereafter, T- and B-cell dependent specific immunity develops allowing the clearance of *Salmonella* microorganisms from the tissues and the establishment of long-lasting acquired immunity to re-infection. The increased resistance that develops after primary infection/vaccination requires T-cells cytokines in addition to opsonizing antibody (Mastroeni et al., 2001). Nevertheless, for reasons that are not completely understood, seroconversion and/or the presence of detectable T-cell memory does not always correlate with the development of acquired resistance to infection (Mastroeni et al., 2001).

The best vaccine against *S.* Typhimurium prevents: (1) colonization; (2) shedding of the bacteria in the environment; (3) the development of carriers; and (4) clinical salmonellosis and promotes elimination of *Salmonella* bacteria from the infected porcine host (Haesebrouck et al., 2004). However, at this moment, as in poultry, vaccination against *Salmonella* in pigs seems only to reduce the infection pressure and is effective, especially in addition to other preventive measures taken at farm level and at the abattoir. In a more realistic approach, a vaccine should be able to: (1) prevent clinical symptoms, (2) reduce shedding by infected pigs and hence spreading to other pigs and (3) increase the threshold for infection of susceptible pigs with *S.* Typhimurium. An efficient vaccine should therefore contribute to the break of the infection chain (Haesebrouck et al., 2004). Whole-cell killed vaccines and subunit vaccines are used in the prevention of *Salmonella* infection

in animals and in humans with variable results (Mastroeni & Ménager, 2003; Mastroeni & Maskell, 2006). Live *Salmonella* vaccines resultant of chemical or u.v. mutagenesis demonstrated to be immunogenic and protective, and are still in use, in spite of the need for repetitive parenteral administration (Mastroeni et al., 2001). Progress in the knowledge of the genetics of *Salmonella* virulence and modern recombinant DNA technology offers the opportunity to introduce multiple defined attenuating and irreversible mutations into the bacterial genome. This has lately allowed the development of *Salmonella* strains devoid of significant side effects but still capable of inducing solid immunity after single oral administration. Live attenuated *Salmonella* vaccines have been used for the expression of heterologous antigens/proteins that can be successfully delivered to the immune system (Mastroeni et al., 2001; Mastroeni & Ménager, 2003). Hence, live vaccine strains offer an improved protection against *Salmonella* infections compared to inactivated vaccines, possibly due to the more marked cellular immune response and the induction of mucosal IgA production (Boyen et al., 2008; Denagamage, O'Connor, Sargeant, Rajic, & McKean, 2007; Haesebrouck et al., 2004; Mastroeni et al., 2001). The evidence available suggests that *Salmonella* vaccines are related with reduced prevalence in swine at or near harvest. For instance, vaccination with a live modified *S.* Choleraesuis vaccine twice at 3 and 16 weeks of age was effective in reducing *Salmonella* prevalence in ileocecal lymph nodes at slaughter from 7.2% (unvaccinated barns) to 0.6% (vaccinated barns) (Denagamage et al., 2007; Maes et al., 2001). Moreover, effective vaccine-induced immunity requires control bacterial growth at each focus of infection and must hinder the redistribution of *Salmonella* to new foci (Mastroeni & Ménager, 2003). Knowledge of the anatomical sites where protective immunity must operate seems to be relevant to vaccine design (Mastroeni & Ménager, 2003). However, conclusions seem to be based on studies with design and reporting deficiencies that could potentially indicate biases with the outcome (Denagamage et al., 2007). In spite of the great recent advances in the development of *Salmonella* vaccines, a large proportion of the work has been conducted in laboratory rodents, and more research in other animal species seems to be required to improve effectiveness (Denagamage et al., 2007; Mastroeni et al., 2001).

3.2.1. Interactions of other microbial agents with Salmonella

Bacterial (*Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*) and viral (hog cholera virus, porcine reproductive and respiratory syndrome virus (PRRSV), Aujeszky's disease virus) infections can result in immunodeficiency in pigs (Segalés et al., 2004). Additionally, some of these and also other swine pathogens (porcine parvovirus, swine influenza virus, African swine fever virus) are capable of replicate in different immune cells and damage their function. These infections may facilitate colonization by *Salmonella*, increased shedding or even higher mortality rates in pigs (Boyen et al., 2008).

3.3. Herd-level risk factors

It has been recognized that there are some herd level factors that could have an influence in *Salmonella* positivity. Size of the herd or type of pen partitions and wall separations, floor materials pens, management procedures such as continuous or all-in/ all-out production systems, feeding practices, including pH of feed and type of feeding, as well as conditions associated with general hygiene and health status, have been identified as the main factors related with a variable prevalence of *Salmonella* infection in swine farms from different countries (Belœil et al., 2004; Creus, Pérez, Peralta, Baucells, & Mateu, 2007; Davies, 2011; European Food Safety Authority, 2011a; Fosse, Seegers, & Magras, 2009; García-Feliz, Carvajal, Collazos, & Rubio, 2009; Lo Fo Wong et al., 2004; Nollet et al., 2004; Vico et al., 2011). For example, the administration of a coarsely ground meal diet to finisher pigs significantly reduced *Salmonella* prevalence compared with pelleted feed (García-Feliz et al., 2009; Lo Fo Wong et al., 2004), and the addition of acids in feed during all the fattening period would be effective to reduce the spread in pig farms (Creus et al., 2007). Biosecurity, mainly related with rodents and bird control, as the quality of drinking water, are important and frequently referred factors (Vico et al., 2011). The size of the herd influence is not completely clear, as some studies results point to the odds of pens being positive to *Salmonella* increased as the holding size enlarged (European Food Safety Authority, 2011a; García-Feliz et al., 2009), however, an increase in herd size does not necessarily mean an increase in pig density at the pen level (Lo Fo Wong et al., 2004). In fact, larger operations might have the resources necessary for the implementation of effective biosecurity measures and good manufacturing practice and an increased risk for *Salmonella* in small-to-moderate-size herds (<800 finishers), compared to large herds, supports this assumption (Lo Fo Wong et al., 2004). The application of good hygiene and biosecurity practices in herds, notably with respect to cleaning and disinfection procedures and high hygiene standard of clothes, and limiting the mixing of pig batches, may reduce the contamination pressure at slaughter (Fosse et al., 2009). Given the diversity of results within MS, EU recommends further studies identifying more closely the risk factors, taking into account the *Salmonella* prevalence and the characteristics of the breeding pig population, regarding the design and implementation of national *Salmonella* control programs (European Food Safety Authority, 2011a).

3.4. Current monitoring and control programs

The implementation of monitoring programs and harmonization of control measures at harvest and post-harvest, are being used worldwide to prevent non-typhoidal *Salmonella* infections in humans from contaminated pork and sustain consumer

confidence (Boyen et al., 2008; Chen, Wang, Chen, Yang, & Yeh, 2006; Hamilton, Smith, & Pointon, 2007; Mossel, Morris, Struijk, Cowden, & Browning, 2003; Ojha & Kostrzynska, 2007; Padungtod & Kaneene, 2006; Rajic et al., 2007). There are three levels of *Salmonella* control measures: the pre-harvest level (on farm), the harvest level (transport to and procedures in the abattoir and the post-harvest level (cutting, processing, retail and food preparation at home). National monitoring and control programs at the farm level are generally conducted in the European countries, according to Regulation EC 2160/2003 (Asai et al., 2002; European Food Safety Authority, 2006; Hamilton et al., 2007; Rajic et al., 2007). Scandinavian countries have been the only to acquire a low prevalence status by EFSA. In Sweden, pre-harvest and harvest monitoring programs are being applied on both a compulsory and a voluntary basis, using mainly bacteriological isolation to evaluate *Salmonella* contamination (Ball, Magowan, Taylor, Bagdonaite, & Madden, 2011; European Food Safety Authority, 2006). The German, British, Irish and Danish programs are founded on serological testing of meat juice samples collected at the abattoir, accordingly classifying the pig herds taking account to their assessed risk of carrying *Salmonella* into the slaughter plant (Alban, Stege, & Dahl, 2002; Ball et al., 2011; Davies et al., 2004; European Food Safety Authority, 2006; Merle et al., 2006; Merle et al., 2011; Nielsen et al., 2001; Wegener et al., 2003). Belgian and Dutch monitoring programs are equivalent, but the serological testing is currently performed on blood or serum samples collected on the farm (Bollaerts et al., 2008; Boyen et al., 2008; European Food Safety Authority, 2006; Hanssen, Swanenburg, & Maassen, 2007). In these countries, farmers with herds included in the classification with the highest risk of introducing *Salmonella* into the abattoir have been supported by the national governments to reduce the load of their herd (European Food Safety Authority, 2006). In Portugal as in Spain, in spite to high results to both countries at the slaughter and herd level (European Food Safety Authority, 2008, 2009), a *Salmonella* National Control Program is still forthcoming (Arguello, Carvajal, Collazos, García-Feliz, & Rubio, 2011; García-Feliz et al., 2008).

3.5. The EU assessment: the most frequent serotypes in humans, pigs and pork

To protect consumers from this food-borne zoonosis, the EU has recently carried out a combined approach to food safety “from the farm to the fork”. The procedure involves risk assessment (e.g. data collection, analysis, recommendations) and risk management (e.g. legislative measures, targets for reduction) measures including EU Member States (MS), European Commission, European Parliament, EFSA, the European Centre for Disease Prevention and Control (ECDC) and economic operators (European Food Safety Authority, 2008, 2009, 2010b, 2011a, 2011b, 2012a, 2012b).

Recent EU 2010 data, demonstrate that, as in previous years, the two most commonly reported *Salmonella* serovars were *S.* Enteritidis and *S.* Typhimurium, representing 45.0% and 22.4%, respectively, of all described serovars in human confirmed cases. Monophasic *S.* Typhimurium was the fourth most frequently reported serovar in human cases (1.5%) and was related to three human outbreaks (0.9% of all *Salmonella* outbreaks). The outbreaks occurred in Germany and were related with pig meat or pork buffet meals (European Food Safety Authority, 2012a).

The EU 2010 serovar results from animals and animal products show that monophasic *S.* Typhimurium was the second most common serovar in pigs (9.3%) and the third in pig meat (7.4%). As in 2008 and 2009, *S.* Typhimurium (30.7%) and *S.* Derby (16.2%) were the most frequently isolated serovars in pig meat. As observed in pig meat, in swine *S.* Typhimurium was by far the most recurrently reported serovar (28.6%).The third and fourth serovars, *S.* Derby (5.7%) and *S.* Choleraesuis (5.2%), were the most frequently reported serovars in Estonia and Romania, respectively (European Food Safety Authority, 2012a). The predominance of *S.* Choleraesuis in Romania is of interest as this serovar has been eliminated from most Western European herds (Boyen et al., 2008; Chang et al., 2005; Chiu et al., 2004; Nollet et al., 2006). The relative importance of the most frequently isolated serotypes in humans and swine, underlining the contribution of *S.* Typhimurium, is demonstrated by Figure 1.

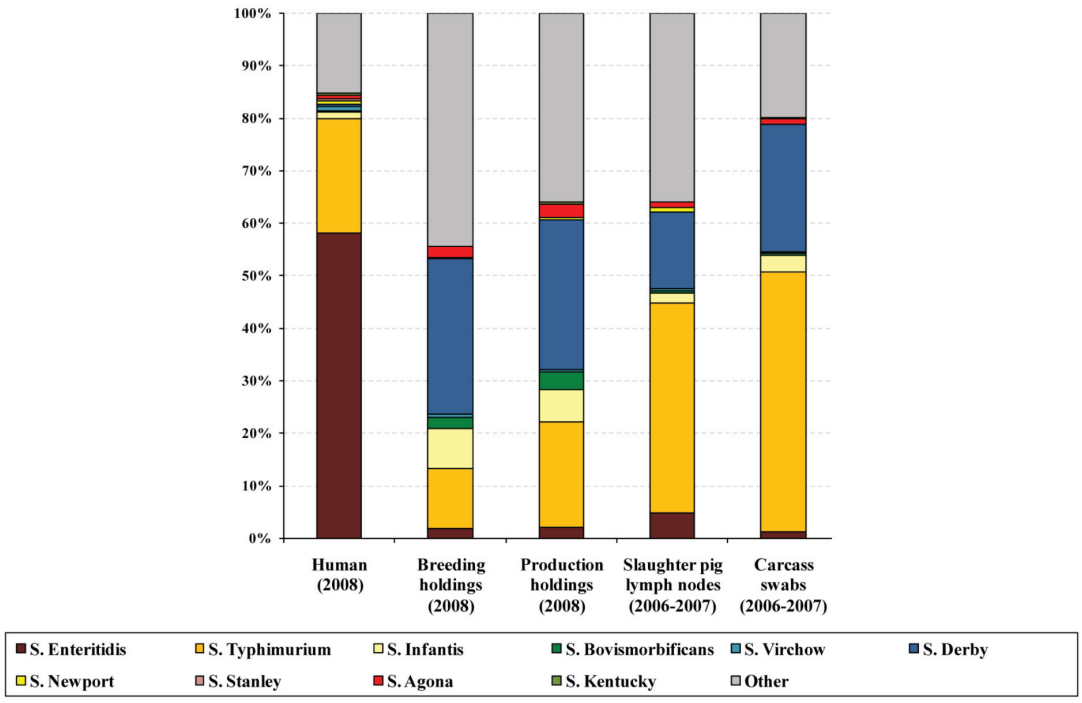

Figure 1. Comparison of the *Salmonella* serovar distribution in humans and animal sources in the EU. *Salmonella* EU baseline survey, 2008 (adapted from European Food Safety Authority, 2011a).

3.5.1. Portuguese swine

Based on a recent EFSA report (European Food Safety Authority, 2009), the prevalence of *Salmonella*-positive swine-breeding holdings and swine-production holdings in the European Union was 28.7% and 33.3% respectively. The prevalence in Portugal was found to be above the average EU-level both in breeding (45.5%) and production holdings (43.3%), in a similar pattern to some other MS (Figure 2). Regarding serotypes, the predominant serovars were *S.* Derby and *S.* Typhimurium both in breeding (29.6% and 28.5%) and production holdings (25.4% and 20.1%) (European Food Safety Authority, 2009).

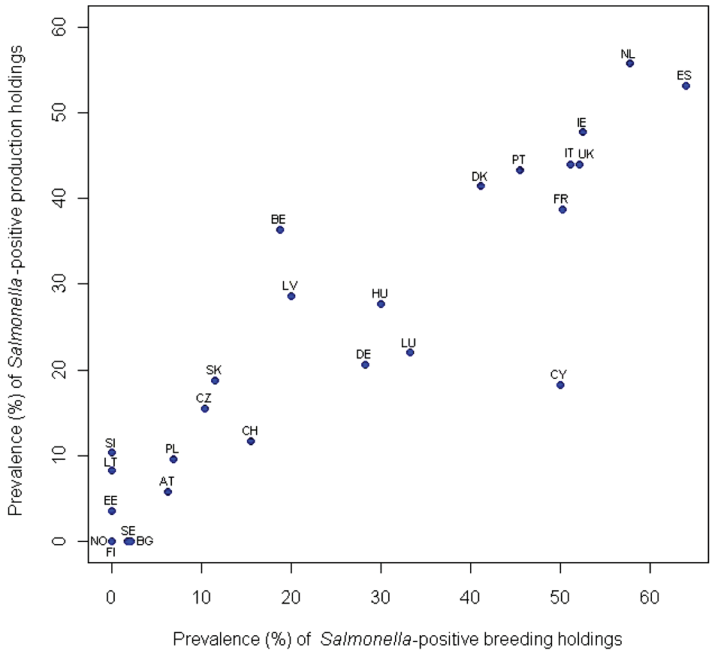

Figure 2. Scatter diagram of the prevalence of *Salmonella*-positive breeding holdings versus the prevalence of *Salmonella*-positive producing holdings in the EU member states (from European Food Safety Authority, 2011c). AT-Austria; BE-Belgium; BG-Bulgaria; CH-Switzerland; CY-Cyprus; CZ-Czech Republic; DE-Germany; DK-Denmark; EE-Estonia; ES-Spain; FI-Finland; FR-France; HU-Hungary; IE-Ireland; IT-Italy; LT-Lithuania; LU-Luxembourg; LV-Latvia; PL-Poland; PT-Portugal; NL-The Netherlands; NO-Norway; SE-Sweden; SL-Slovenia; SK-Slovakia; UK-The United Kingdom.

In accordance with a EU baseline survey, the observed prevalence of *Salmonella* in slaughter pigs for the EU as a whole was 10.3% for lymph nodes (Portugal - 23.4%) (Table 2) and 8.3% for carcasses (Portugal was not included in that part of the survey) (European Food Safety Authority, 2008).

Table 2. Observed prevalence of slaughter pigs infected with Salmonella in lymph nodes, in the EU and Norway, 2006-2007 (from European Food Safety Authority, 2008)

		***Salmonella* spp.**		***S.* Typhimurim**		***S.* Derby**		**Serovars other than *S.* Typhimurim and *S.* Derby**	
Member State	**N total**	**% prev.**	**CI**	**% prev.**	**CI**	**% prev.**	**CI**	**% prev.**	**CI**
Austria	617	2.0	1.1-3.6	0.7	0.2-2	0.3	0.1-1.1	1.1	0.5-2.3
Belgium	601	13.9	9.8-19.3	7.8	5.3.11.5	1.3	0.4-3.6	4.9	3.0-7.9
Bulgaria	176	16.7	8.1-31-4	1-8	0.6-4.9	4.9	1.3-16.4	10.1	4.9-19.7
Cyprus	359	12.4	10.1-15.2	1.0	0.8-1.3	0		11.5	9.1-14.5
Czech Republic	654	5.8	3.8-8.9	1.6	0.8-3.3	1.4	0.5-4.1	2.7	1.6-4.5
Denmark	998	7.7	5.5-10.7	4.5	3.4-5.9	1.3	0.8-2.2	2.0	1.4-3.0
Estonia	420	4.7	2.3-9.4	1.1	0.6-2.1	0		3.8	1.7-8.3
Finland	419	0		0		0		0	
France	1.163	18.1	16-20.5	7.1	5.4-9.5	6.5	5.6-7.4	4.5	3.2-6.3
Germany	2.567	10.9	8.8-13.5	6.1	4.7-7.8	1.2	0.8-1.8	4.3	3.4-5.5
Greece	345	24.8	18-33.2	3.4	1.6-7.1	3.8	1.6-8.8	17.2	11.7-24.6
Hungry	658	9.3	5.3-15.8	2.9	1.4-5.9	1.5	0.4-5.2	4.7	2.9-7.6
Ireland	422	16.1	15.6-16.7	9.1	9-9.2	2.4	2.3-2.5	3.6	2.0-6.4
Italy	709	16.5	14.1-19.1	1.6	0.9-2.6	5.4	3.8-7.7	9.6	7.7-12.1
Latvia	392	5.6	3.3-9.1	0.3	0.1-2	1.9	0.6-6	3.4	1.7-6.6
Lithuania	461	1.8	0.8-3.9	1.3	0.5-3.8	0		0.5	0.2-1.5
Luxembourg	313	22.4	12.7-36.4	16.1	8.8-27.6	1.5	0.7-2.8	4.0	1.6-9.6
Poland	1.176	5.1	3.7-6.9	1.4	0.8-2.5	0.1	0-0.2	3.5	2.5-4.9
Portugal	658	23.4	19.4-28	8.4	6.1-11.5	2.5	1.3-4.7	12.1	10.3-14.2
Slovakia	385	4.8	2.6-8.9	0.8	0.3-2.1	1.1	0.4-2.7	3.6	1.8-6.8
Slovania	431	6.2	4.2-9.1	0.7	0.2-2	0.6	0.1-2.6	5.1	3.4-7.5
Spain	2.619	29.0	24.9-3.5	10.6	8.6-13.1	2.8	1.8-4.3	16.1	13.5-19.1
Sweden	394	1.3	1.2-1.5	1.2	0.5-2.7	0		0.5	0.3-0.5
The Netherlands	1.087	8.5	7.3-9.8	4.9	4.7-5	1.3	0.8-2.1	2.1	1.4-3.2
The United Kingdom	639	21.2	17.8-25	13.8	11.9-15.8	4.8	3.6-6.3	3.8	2.5-5.5
EU	18.663	10.3	9.2-11.5	4.7	4.1-5.3	2.1	1.8-2.6	5.0	4.4-5.7
Norway	408	0.3	0.04-1.6	0.3	0.04-1.6	0		0	

A previous study carried out in a Portuguese swine abattoir identified *Salmonella* in 19% and 13% lymph node samples and carcasses, respectively. The most frequent serotypes were *S.* Typhimurium (48%) and *S.* Rissen (30%) (Vieira-Pinto et al., 2005), accordingly with the EU baseline survey data (Table 3).

Table 3. Frequency distribution of *Salmonella* serovars in lymph nodes samples of slaughter pigs in EU and Norway, 2006-2007, data from Portugal (adapted from European Food Safety Authority, 2008)

Portugal	Lymph node samples with serovars	N	%
	S. Typhimurium	57	36.5
	S. Rissen	22	14.1
	S. 4,[5],12:i:-	17	10.9
	S. Derby	17	10.9
	S. Enteritidis	9	5.8
	S. Give	7	4.5
	S. Newport	7	4.5
	S. Anatum	6	3.8
	S. Agona	5	3.2
	S. Bovismorbificans	2	1.3
	S. Eboko	1	0.6
	S. Gaminara	1	0.6
	S. Havana	1	0.6
	S. Infantis	1	0.6
	S. Mbandaka	1	0.6
	S. Ohio	1	0.6
	S. Panama	1	0.6
	Total isolates	156	

4. *Salmonella* and antimicrobial resistance

Antibiotics — naturally-occurring, semi-synthetic and synthetic compounds with antimicrobial activity — are used in human and veterinary medicine to treat and prevent disease, and for other purposes, including growth promotion in food animals (Phillips et al., 2004). Antibiotic resistance is as ancient as antibiotics, protecting antibiotic-producing organisms from their own products, and other originally susceptible organisms from their competitive attack in nature (Amyes, 2010; Phillips et al., 2004). All antibiotics can select spontaneous resistant mutants and bacteria that have acquired resistance by transfer of genetic material from other bacteria. These antimicrobial-resistant phenotypes can be achieved in microorganisms by chromosomal DNA mutations, which modify existing bacterial proteins, through alteration, which can create mosaic proteins and/or as a result of transfer and acquisition of new genetic material between bacteria of the same or different species or genera (van Hoek et al., 2011). These resistant variants, as well as species that are inherently resistant, can become dominant and spread in host-animal populations (Amyes, 2010; Hancock, 2012). The more an antibiotic is used, the more

likely are resistant populations to develop among pathogens and commensal bacteria of an increasing number of animals in an exposed population (Jansen, Van der Bruggen, Verhoef, & Fluit, 2006; Johnsen et al., 2009; van Hoek et al., 2011). However, there is great diversity: whereas some bacteria very rapidly develop resistance, others remain susceptible (Gould, 1999; Phillips et al., 2004). Over the past six decades, bacterial populations have replied to the selective pressure of antimicrobial drugs by developing resistance to all commercially existing agents (Johnsen et al., 2009; Levin, 2001). The population dynamics of antimicrobial resistance depend upon the substances administered; resistance is also influenced by a number of other factors, not least: the availability of pre-existing resistance genes, the exchangeability of the resistance genes and their functional activity in different bacterial hosts, and the selective pressure (Schwarz, Kehrenberg, & Walsh, 2001). Obviously, if antibiotics are present within the environment, there is strong selective pressure for the spread of resistance and those factors that promote the spread and gene transfer, for instance, is also more likely in environments where bacteria are in close proximity to each other and in relatively high density, such as the gut and oral cavity (van Hoek et al., 2011).

Within *Salmonella* several multidrug-resistant (MDR) strains seem to have gained relative advantages as they have managed to spread rapidly in some animal and human populations, for example *S.* Newport in the USA, *S.* Typhimurium DT104 in Europe, and the monophasic variant 4,5,12:i:- of *S.* Typhimurium which is currently increasing in Europe, associated with pigs and pork (Butaye et al., 2006; European Food Safety Authority, 2012a, 2012b; Hauser et al., 2010; Hopkins et al., 2010). Within *Salmonella*, especially *Salmonella enterica* serotype Typhimurium, these MDR strains cause particular concern because of their increasing prevalence in humans becoming a worldwide health problem (Butaye et al., 2006; Parry & Threlfall, 2008; Shahada, Amamoto, Chuma, Shirai, & Okamoto, 2007; Shahada, Sugiyama, Chuma, Sueyoshi, & Okamoto, 2010).

The antimicrobial agents' widespread use in pigs during rearing created a selective pressure that may have contributed to the occurrence and dissemination of these MDR strains, which can be transmitted to humans through food products, particularly those of animal origin (Botteldoorn et al., 2004; Carattoli, 2008; Daly & Fanning, 2000; Threlfall, 2002). The campaign against what has been considered excessive clinical use has been generally directed at human and veterinary medicine. However, there has been also an intensive opposition on the food producing animals' use of antibiotics, based upon the assumption that is imprudent and may act as an important source of resistance in bacteria affecting humans (European Commission, 1998, 1999; Levy, 1984, 2001; Threlfall, 2002; Witte, 1998; World Health Organization, 2001). In Europe, since the first harmonization by Directive 70/524 until Regulation (EC) No 1831/2003 on additives for use in animal

nutrition, there was, since 2006, a total banning of antibiotic growth promoters as a precaution (Castanon, 2007). The decreasing of the antibiotics used for animal production, and consequently, the reduction of the risk of transferring to human of bacterium with antibiotic-resistant genes, is the main expected result from this banning (Castanon, 2007). Otherwise, data suggested that the growth-promoter ban had determined an increase in infections and, consequently, a considerable growing through the use of therapeutic antibiotics for food animals in Europe, nevertheless, a reduced overall antimicrobial use in animals (McEwen, 2009). In Sweden, it was reported that as a result of the previous national banning in 1986 and an effort on disease prevention and correct use of antimicrobials, the total use of antibacterial drugs to animals diminished by approximately 55% in the period 1986-1999, and a comparatively low prevalence of antimicrobial resistance has been preserved (Wierup, 2001a). Recommended voluntary guidelines include orientation for prudent use among veterinarians, as well as information to farmers on cautious use for antibiotics that are sold over the counter (Höjgård & Vågsholm, 2010). For example, in Sweden, it is considered a good veterinary practice that drugs should only be prescribed to individual animals after a clinical and laboratory examination identifying the disease-causing agent. Another recommendation is that narrow spectrum antibiotics should be preferred to broad-spectrum drugs (Höjgård & Vågsholm, 2010). However, in antimicrobial free swine production systems, MDR *S.* Typhimurium strains were also detected, regardless of the absence of antimicrobial selection pressure (Thakur et al., 2007).

4.1. Resistance phenotypes

Surveillance data demonstrated an increase in overall antimicrobial resistance among salmonellae from 20%-30% in the early 1990s to as high as 70% in some countries after the turn of the century (Boyen et al., 2008). The resistance rate, however, varies with distinct serotypes and different antibiotics, *S.* Enteritidis, one of the most prevalent *Salmonella* serotypes, is relatively more susceptible to antimicrobial agents than are other serotypes (Boyen et al., 2008). A much higher rate of resistance was found in *S.* Typhimurium, another globally prevalent serotype (Su et al., 2004). In the early 1990s, a distinct MDR strain of phage type 104 (DT104) *S.* Typhimurium strain was isolated and found to be simultaneously resistant to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline, which corresponds to an ACSSuT phenotype (Boyen et al., 2008; Helms, Ethelberg, & Mølbak, 2005; Mølbak et al., 1999; Threlfall, 2002). Phage type DT104 is frequently isolated from pigs or pork (Gebreyes et al., 2004; Threlfall, 2000).

In a recent EU data registered for *Salmonella* isolates from pigs (European Food Safety Authority, 2012b), resistance levels were 57% for tetracyclines, 55% for ampicillin and 59% for sulfonamides. Ciprofloxacin and nalidixic acid resistance levels were low, at 3% and 2% respectively, whereas the level of resistance to cefotaxime about 0.8%. Regarding pig meat, the resistance to tetracyclines (50%), ampicillin (47%) and sulfonamides (52%) was also common in *Salmonella* spp. isolates. Resistance to ciprofloxacin and nalidixic acid was 5% and 4%, respectively, and cefotaxime resistance equaled 0.2% (European Food Safety Authority, 2012b). Over the years 2005-2010, a relatively stable situation in resistance has been observed in *Salmonella* spp. isolates from pigs in the EU (European Food Safety Authority, 2012b). Regarding salmonellosis in EU, resistance in human *Salmonella* isolates, especially *S.* Typhimurium, was very high for ampicillin (64%), tetracyclines (58.5%) and sulfonamides (57.2%) and high for streptomycin (44.1%). Resistance to these antimicrobials in isolates from monophasic *S.* Typhimurium was extremely high, in all cases above 80% (European Food Safety Authority, 2012b).

Due to the spread of resistance to conventional antibiotics, the currently recommended drugs of choice for treatment of salmonellosis in humans are fluoroquinolones and third-generation or extended-spectrum cephalosporins, but therapy may be also complicated by the fact that antimicrobial resistance in *Salmonella* isolates from these infections has become increasingly common (Chang et al., 2005; Chiu et al., 2004; Stoycheva & Murdjeva, 2006; Su et al., 2004). According to EU data, resistance to these clinically important antimicrobials was still relatively low, namely ciprofloxacin (8.6%) and cefotaxime (1.0%). Noteworthy, resistance to quinolones (ciprofloxacin and nalidixic acid) was higher in *S.* Enteritidis (9.3%, 18.7%) isolates than in *S.* Typhimurium isolates (4.7%, 8.9%) (European Food Safety Authority, 2012b).

Recently, a new clonal group with resistances to ampicillin, streptomycin, sulfonamides, and tetracycline (ASSuT) was identified in Italy, United Kingdom and Denmark among human and animal strains of *S.* Typhimurium and its monophasic variant, suggesting its circulation in different European countries (Graziani et al., 2008; Lucarelli et al., 2010; Lucarelli et al., 2012).

4.2. Resistance determinants and transmission

Antibiotic resistance is the best-known example of rapid adaptation of bacteria to a new ecosystem (Carattoli, 2001). The capability of bacteria to develop their ecological niche, also in the presence of certain antibiotics, can be elucidated by the acquisition of resistance genes by horizontal gene transfer and/or by the accumulation of point mutations leading to the alteration of existing genes (Carattoli, 2001; van Hoek et al.,

2011). Several studies on bacterial pathogens of human and animal origin concluded that MDR is a consequence of horizontal gene transfer, mediated by bacteria transformation, transduction and conjugation (Carattoli, 2001).

Nowadays, a worrying trend is the presence of genetic elements that co-integrate antibiotic resistance and virulence determinants, compromising the therapeutic options in cases of invasive *Salmonella* infections (Fluit, 2005). Indeed, a variety of transposable elements has been recently identified contributing to the dissemination of relevant antimicrobial resistance genes in *Salmonella* (Lucarelli et al., 2012; Miriagou, Carattoli, & Fanning, 2006; Stellwagen & Craig, 1998). It is well established that the distribution of antimicrobial resistance is often mediated by mobile genetic elements (MGEs), such as plasmids and/or transposons and/or insertion sequences (Lucarelli et al., 2012) (Table 4). Indeed, several of the antibiotic resistance genes observed in Gram negative

Table 4. Characteristics of the most important MGEs (from Manageiro, 2011)

Gene Transfer Element	Characteristics of DNA transfer elements
Plasmid	Plasmids are transferable genetic elements capable of autonomous replication within a suitable host. Plasmids can be either *self-transmissible* (conjugative) or *mobilisable* (non-self-transmissible). Whereas the first group encodes a complete conjugative DNA transfer apparatus (Trafunctions), the second group usually bears only the functions required for initiation of its own transfer DNA rreplication (Mob functions).
Insertion sequences	Insertion sequences (IS) are the simplest transposable elements; by definition, IS carry only the genetic information necessary for insertion functions and no accessory genes (for example, drug resistance). IS elements are small genetic elements that are flnked by short terminal inverted-repeat sequences (IR) of 10-40 bp and are able to insert a multiple sites in target DNA
ISCR	*Insertion Sequence Common Region* (ISCR) elements are IS that have similarities to the *IS91* family in both structure and function. These elements are known to move by a process called rolling-circle replication, and a function of this process is the concomitant movement of additional sequences found upstream of their transposase genes.
Transposon	*Transposons* are genetic elements that physically transpose from one genetic position to another, within the chromosome or plasmid in which they reside. Some transposons carry one or more antibiotic resistance genes in their central regions. *Complex transposons* contain IS with short IR at their termini; undergo replicative transposition *Conjugative transposons*, also called integrated conjugative elements, are integrated DNA elements that exercise thamselves to form covalentely ciosed circular intermediate. This circular intermediate can either reintegrate in the same cell or transfer by conjugation to a recipient and integrate into the recipient's genome.
Integron	Integrons are DNA elements, not self transmissible, with the ability to capture genes, by site-specific recombination. Integrons have an integrase gene (*inf*) to mediate excision and orientation-specific integration of gene cassettes, a nearby recombination site (*attI*), and a promoter, Pc, which ensures expression of the operon. There are three main classes of integrons based upon the type of integrase gene they possess: class 1 and class 2 integrons are the mot common, whereas class 3 are rare.
Gene cassette	*Gene cassettes* are genetic elements that may exist as free, circular, non-replicting DNA molecules when moving from one genetic site to another, but which are normally found as linear sequences that constitute part of a larger DNA molecule, such as a plasmid or bacterial chromosome. The genes carried on gene cassettes usually lack promoters and are expressed from a promoter on the integron.
Bateriophage	*Bacteriophage* (phage) are obligate intracellular parasites that multiply inside bacteria by making use of some or all of he host biosynthetic machinery (i.e., viruses that infect bacteria). They mediate the transfer of resistance genes.

microorganism as *Salmonella* are part of a gene cassette inserted in an integron (Hall & Collis, 1998; Rowe-Magnus & Mazel, 2002). These gene cassettes contain genes conferring resistance to a range of antimicrobial agents, including aminoglycoside, beta-lactams, chloramphenicol and trimethoprim, as well as genes that confer resistance to antiseptics and disinfectants (Hall & Collis, 1998; Rowe-Magnus & Mazel, 2002).

The evaluation of drug resistance at a molecular level is, therefore, an important tool for understanding the participation of genetic elements for the expression of resistance and its possible transfer among bacteria (Fluit, Visser, & Schmitz, 2001; Shahada et al., 2010; van Hoek et al., 2011). Moreover, in order to control the spread of resistance, is important to associate this evaluation with the study of the ecology of the environments in which spread is likely (van Hoek et al., 2011).

SECTION II

AIMS OF THE THESIS

1. Aims of the thesis

Salmonellae are accepted as an important source of human infections being mainly transmitted through food of animal origin, and pork products are recognized as a relevant source of salmonellosis (Berends et al., 1998a, 1998b; Boyen et al., 2008; De Busser et al., 2011; Delhalle, Saegerman, Farnir, et al., 2009; European Food Safety Authority, 2011b; Gebreyes et al., 2004; Valdezate et al., 2005). Particular MDR *Salmonella* clones mainly associated with asymptomatic swine and pork, have been increasingly involved in human infections (Dionisi et al., 2009; Hauser et al., 2010; Lucarelli et al., 2010; Switt, Soyer, Warnick, & Wiedmann, 2009).

According to recent data, Portugal was found to be above the average EU-level of *Salmonella* prevalence both in breeding and production pig holdings and in slaughter pigs (European Food Safety Authority, 2008, 2009). In Portugal, swine production, abattoir stages and post-harvest pork meat production chain are insufficiently accessed (Antunes, Mourão, Pestana, & Peixe, 2011; Vieira-Pinto et al., 2005; Vieira-Pinto et al., 2006) and the contribution of slaughter and deboning operations in the contamination of meat with *Salmonella* remains to be studied. Furthermore, there is a lack of characterization of MDR clones in slaughtered swine. Additionally, in spite of the specificity of EU Food law applicable to abattoir and meat plants, there is a general lack of information about professional training for slaughterhouses and deboning room's workers. The aim of this thesis was to fill the gaps mentioned above by increasing the knowledge about *Salmonella* in Portuguese slaughter swine and its impact in food chain and public health, enlightening meat handler's participation in the overall process.

Specific **aims** of the studies included were to:

a) Identify the occurrence of *Salmonella* in slaughter swine from different abattoirs.
b) Isolate *Salmonella* in different sources unveiling routes of cross-contamination in the abattoir environment, characterizing the genetic relatedness of the isolates.
c) Investigate antimicrobial resistance in strains obtained from swine, carcasses, meat and meat handlers, characterizing resistance phenotypes.
d) Identify genetic determinants of resistance, characterizing resistance genotypes.
e) Evaluate and compare the level of general knowledge and practice in meat handlers from slaughter houses and meat plants, as a contribution to the comprehension of the participation of meat handlers in *Salmonella* MDR dissemination.

This study was conducted in abattoirs and meat plants of the north region of Portugal, and the development of the experimental work and respective results are present in the following chapters under the form of research papers.

SECTION III

RESULTS

Chapter 1. From *Salmonella*-carrier pigs to contaminated pork: from farm to fork

Paper I. *Salmonella* cross-contamination in swine abattoirs in Portugal: carcasses, meat and meat handlers.

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Salmonella cross-contamination in swine abattoirs in Portugal: Carcasses, meat and meat handlers

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ABSTRACT

In this study the occurrence of *Salmonella* in swine, pork meat and meat handlers along with their clonal relatedness is evaluated at abattoir level. Samples from the lymph nodes, carcass surface and meat of 100 pigs and 45 meat handlers were collected in eight abattoirs (July 2007–August 2008). *Salmonella* isolates were serotyped and genotyped by pulsed-field gel electrophoresis (PFGE). From the pigs tested, 42 produced at least one positive sample. A relatively high frequency of *Salmonella* occurrence was found in the ileoceacal lymph node samples (26.0%), followed by carcass (16.0%) and meat samples (14.0%). However, ileoceacal lymph nodes that test positive for *Salmonella* are not found to be a predictor of positive test results further on in the process. Besides the slaughterhouse environment, meat handlers were identified as a possible source of subsequent contamination, with 9.3% of the sample testing positive. Diverse *Salmonella enterica* serotypes were detected, mainly *S.* Typhimurium and the monophasic variant *S.* 4,[5],12:i:-, but also *S.* Derby, *S.* Rissen, *S.* Mbandaka, *S.* London, *S.* Give, *S.* Enteritidis and *S.* Sandiego, in total corresponding to 17 PFGE types. Our results demonstrate that besides a high level of *Salmonella* swine contamination at pre-harvest level, slaughtering, dressing, cutting and deboning operations are contributing to the occurrence of clinically relevant clones (e.g. *S.* Typhimurium DT104 and *S.* 4,[5],12:i:-) in pork products. This study also highlights the possibility of an ongoing *Salmonella* community being spread by abattoir workers.

1. Introduction

Salmonella is a significant cause of food-borne illness in humans with 108 614 reported cases in the European Union in 2009, with farm animals also being affected. Food of animal origin is identified as being the main vehicle for transmission to humans (EFSA, 2011). In pigs, the most common serotypes are *S.* Typhimurium and *S.* Derby, with pork products recognized as a being a source of human salmonellosis (Berends et al., 1998a,1998b; De Busser et al., 2011; Delhalle et al., 2009; EFSA, 2006, 2008a,2008b, 2009; Gebreyes et al., 2004; Valdezate et al., 2005). Other sources associated with pork have arisen from the emergence of clinically relevant multidrug resistant clones, namely *S.* 4,[5],12:i:-, *S.* Typhimurium DT104 and *S.* Rissen, as has been pointed out by several authors (Antunes et al., 2006, 2011; Dionisi, et al., 2009; Hauser et al., 2010; Lucarelli et al., 2010; Soyer et al., 2009; Switt et al., 2009). Swine under intensive production conditions are usually *Salmonella*-positive, as they frequently suffer oral infection, becoming asymptomatic carriers via the tonsils, intestine and associated lymph nodes (Fedorka-Cray et al., 2000). Based on a recent EFSA report (EFSA, 2009), the prevalence of *Salmonella*-positive swine-breeding holdings and swine-production holdings in the European Union was 28.7% and 33.3% respectively. The prevalence in Portugal was found to be above the average EU-level both in breeding (45.5%) and production holdings (43.3%), in a similar pattern to some other Member States (MS). The observed prevalence of *Salmonella* in slaughter pigs for the EU as a whole was 10.3% for lymph nodes (Portugal—23.4%) and 8.3% for carcasses (Portugal was not included in this part of the baseline survey) (EFSA, 2008a).

Despite studies being conducted that evaluate the occurrence of *Salmonella* in the lymph nodes and carcasses of slaughtered swine, the linkage between the different slaughter line operations and the contamination of the final product is poorly elucidated (Berends et al., 1998a,1998b; Botteldoorn et al., 2004; De Busser et al., 2011; Vieira-Pinto et al., 2005). In Portugal, the primary production,

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slaughterhouse stages and post-harvest pork meat production chain have rarely been studied (Antunes et al., 2011; Vieira-Pinto et al., 2005, 2006) while the involvement of slaughter operations in the contamination of meat with *Salmonella* has been insufficiently addressed. The aim of this study is to investigate the occurrence of *Salmonella* in swine products and on meat handlers along critical operations of the slaughter line, and to determine clonal relationships between isolates, assessing the dispersion of recovered strains and their involvement in cross-contamination. To this end, samples taken at different stages of the abattoir's operations and pork processing procedure were evaluated: (1) ileocecal lymph nodes immediately after evisceration; (2) carcass swabs at the end of the slaughter line and prior to refrigeration; (3) meat in the cutting and deboning room, 24 h after slaughter; (4) meat handlers responsible for cutting operations.

2. Material and methods

2.1. Samples

Samples were collected from July 2007 to August 2008 in eight abattoirs (identified from A to H) in the north of Portugal during Meat Inspection by the Official Veterinary Services. The abattoirs included in this study processed swine using a similar principal technique, the only notable difference relating to the vertical scalding system used by four abattoirs (B, C, D and G) as compared to the traditional horizontal scalding tank used by the other four. Abattoirs C and D had the highest rate of pigs slaughtered per unit of time, approximately 250/h, compared to100/h in each of the others. In each abattoir one pig was sampled from each batch, from all pigs scheduled to be slaughtered on the selected day. In accordance with the criteria of the European Commission Decision 2006/668/EC, the following animals were excluded from the study: those with live weight of less than 50 kg or more than 170 kg or those submitted for emergency slaughter (Anonymous, 2006). A total of 100 slaughtered pigs were sampled (50 male/50 female) with a medium weight of 77.6 ± 10.0 kg, from 64 intensive production pig farms located in 11 out of the 18 districts of continental Portugal. All the sampled pigs were approved for slaughter by the Official Veterinary Services and none was condemned in a *post-mortem* inspection. Ileocecal lymph nodes, a carcass external surface swab and posterior limb muscular tissue (meat samples) were collected from each pig. A total of 345 samples were analyzed (300 pig samples and 45 hand samples, as each meat handler performed cutting operations on more than one sampled carcass). The samples were individually packed, registered with the date, abattoir identification and pig or meat handler identification code and kept at a temperature of ≤7 °C during storage and transportation to the laboratory where they were analyzed within a 24 h period.

2.2. Sampling procedure

2.2.1. Lymph nodes

Immediately after evisceration, the stomach-gut package of each pig was separated. In a separate room within the slaughterhouse, the mesenterium between the caecum and the part of the ileum that is closest to the caecum was opened and approximately 15 ileocecal lymph nodes (15 g) were collected.

2.2.2. Carcasses

The carcass samples were obtained immediately after evisceration and before chilling. A surface approximately 10 × 10 cm^2 per site was swabbed with one single abrasive sponge for all the following sites, in accordance with Annex A of ISO standard 17604: hind limb (medial), lateral abdomen (belly), mid-dorsal region (mid-back) and jowl (Anonymous, 2005). A total of 400 cm^2 was swabbed for each carcass sampled. Pre-hydrated sponges with Buffered Peptone Water (Biotrace™ Hydra Sponge 10 ml Neut Buffer 5bg of 20) were used to swab the surface of the carcass in a standardized way: two sites were swabbed with one side of the sponge which was then turned over for the remaining two sites. The sponge was wiped over each sampling site for a total of 10 times in the vertical and 10 times in the horizontal direction. The rules for bacteriological sampling of pig carcasses in slaughterhouses using non-destructive methods were followed, as laid down in Commission Regulation (EC) No 2073/2005 of 15 November 2005, in accordance with Annex A of ISO standard 17604 (Anonymous, 2005).

2.2.3. Meat samples

Meat samples were collected 24 h after slaughter in the deboning room of each abattoir. A fragment of approximately 25 g of muscular tissue from the medial region of the hind limb was collected, during cutting and deboning operations.

2.2.4. Meat handlers' hands

A swab was taken of the back of the hand and area between the fingers of the meat handler at the deboning room immediately after cutting and deboning operations and the collection of samples from the selected carcasses. Pre-hydrated Buffered Peptone Water Swabs (Biotrace™ Redi Swab, 10 ml BPW) were used.

2.3. Microbiological culture method

The samples were analyzed by standard culture methods according to ISO norm 6579:2002 (Annex D010705) applied to *Salmonella* detection in food and animal feedstuffs (Anonymous, 2002). Lymph nodes were dipped into absolute alcohol and air dried to decontaminate the surface before analysis; positive samples correspond to infected lymph nodes and not to contamination with external microorganisms (Anonymous, 2006). Lymph nodes and meat samples were weighed, placed in a sterile container and suspended in diluted (1:10) Buffered Peptone Water (BPW; Merck, Darmstadt, Germany-1.07228) (lymph nodes sample: 15 g + 135 ml BPW; meat sample: 25 g + 225 ml BPW) and homogenized in the stomacher (90 s). The abrasive sponges resulting from carcass swabs and meat handlers' hand swabs were diluted in BPW (10 ml sample + 90 ml BPW). The samples diluted in BPW were incubated at 37 °C for 18 ± 2 h, after which samples were analyzed (Anonymous, 2002).

2.4. Salmonella serotyping and genotyping

Salmonella isolates were serotyped in the Portuguese reference laboratory for *Salmonella,* Laboratório Nacional de Investigação Veterinária (LNIV), according to the Kauffman–White scheme. A PCR assay targeting the *inv*A gene and a specific sequence of DT104/U302 phage type was conducted according to Pritchett et al. (2000). The *S.* Typhimurium monophasic variant (*S.* 4,[5],12:i:-) isolates were confirmed using PCR assays, as previously described (Soyer et al., 2009; Tennant et al., 2010).

Clonality among the isolates was assessed by Pulsed Field Gel Electrophoresis (PFGE) following *Xba*I digestion of genomic DNA according to the standard 1-day protocol of the CDC (CDC, 2002). Genomic DNA from *S.* Braenderup H9812 obtained from the CDC was also restricted with *Xba*I and used as a size standard. PFGE pattern analysis was performed with *Bionumerics* version 3.5 (Applied Maths, Sint-Martens-Latem, Belgium) while the un-weighted pair group method (UPGMA) was used for clustering, with arithmetic averages based on the Dice similarity index. Isolates with a Dice band-based similarity coefficient value >85% with a 1.0% tolerance limit and a 1.0% optimization were considered to belong to the same PFGE type. PFGE types within serotypes were indicated by the capital letter of the name of the serotype followed by a number (e.g. *S.* Typhimurium genotype 1 is indicated as T1).

Table 1
Distribution of *Salmonella* positive pig samples by abattoir.

Abattoir[a]	Pigs sampled (n)	Lymph nodes (%)	Carcasses (%)	Meat (%)	Pigs (%)[b]	Total of positive samples (%)
A	17	6 (35.3)	2 (11.8)	2 (11.8)	10 (58.8)	10(19.6)
B	17	3 (17.6)	3 (17.6)	0	6 (35.3)	6 (11.8)
C	11	5 (45.5)	2 (18.2)	3 (27.3)	6 (54.5)	10 (30.3)
D	9	3 (33.3)	5 (55.6)	4 (44.4)	6(66.7)	12 (44.4)
F	19	4 (21.1)	1 (5.3)	2 (10.5)	6 (31.6)	7 (12.3)
G	9	2 (22.2)	1 (11.1)	0	3 (33.3)	3 (11.1)
H	17	3 (17.6)	2 (11.8)	3 (17.6)	5 (29.4)	8 (15.7)
Total	99	26 (26.3)	16 (16.2)	14 (14.1)	42 (42.4)	56 (18.9)
p value		*0.605*	*0.089*	*0.037*	*0.297*	*0.004*

[a] Abattoir E was excluded from the analysis for the reduced number of pigs sampled (n = 1) and positive samples (n = 1 meat handler).
[b] Positive pigs = at least one positive sample.

2.5. Statistical analysis

The analysis of the results was conducted using the computer software SPSS (SPSS Inc., Chicago, IL; version 17.0). The proportion of positive samples was compared using the Pearson chi-square test (with continuity correction) as appropriate. When the assumptions of the asymptotic method were not met, the exact significance was calculated by applying the Fisher exact test. The 95% confidence intervals (95% CI) of the proportion of positive samples have been estimated according to the Wilson procedure with a correction for continuity (Newcombe, 1998; Wilson, 1927). In all tests, the statistical significance was two-sided and considered significant at $p<0.05$.

3. Results

3.1. Salmonella prevalence

Salmonella was recovered in all abattoirs (abattoir E had a single positive meat handler sample) and among them statistically significant differences in the proportion of total positive samples ($p = 0.004$) and positive meat samples ($p = 0.037$) were found, underlined by the highest results found in abattoirs C and D (Table 1). Overall, *Salmonella* was isolated in 17.6% (60/345) of the samples collected. *Salmonella* was found most frequently in lymph node samples (26/100) followed by carcass samples (16/100) and meat samples (14/100). Regarding meat handlers' contamination, *Salmonella* was isolated in 9.3% of swabs (4/45) (Table 2). There were 42 pigs which returned at least one positive sample, while ten of those presented multiple positive samples (Table 3).

3.2. Salmonella serotypes

Nine different *Salmonella enterica* serotypes were detected in the 60 positive samples: *S.* Typhimurium (53.3%;n = 32) and the monophasic variant *S.* 4,[5],12:i:- (5%; n = 3), *S.* Derby, (18.3%; n = 11), *S.* Rissen (6.6%; n = 4), *S.* Mbandaka (5%; n = 3), *S.* London (5%; n = 3), *S.* Give (3.3%; n = 2), *S.* Enteritidis (1.6%; n = 1) and *S.* Sandiego (1.6%; n = 1). Table 2 presents the distribution of the *Salmonella enterica* serotypes according to the type of samples analyzed. The results, showing the occurrence of *Salmonella* and the serotyping of the isolates, are summarized in Table 3, identifying pigs (from 1 to 100), abattoirs (from A to H) and collecting dates.

Table 2
Distribution of *Salmonella* serotypes by samples.

Identified serotypes	Lymph nodes n (%)	Carcasses n (%)	Meat n (%)	Meat handlers n (%)
S. Typhimurium	11 (42.3)	9 (56.3)	10 (71.4)	2 (50.0)
S. Derby	5 (19.2)	4 (25.0)	2 (14.3)	0
S. Rissen	1 (3.8)	1 (6.3)	1 (7.1)	1 (25.0)
S. 4,[5],12:i:-	2 (7.7)	0	1 (7.1)	0
S. Mbandaka	3 (11.5)	0	0	0
S. London	0	2 (12.5)	0	1 (25.0)
S. Give	2 (7.7)	0	0	0
S. Enteritidis	1 (3.8)	0	0	0
S. Sandiego	1 (3.8)	0	0	0
Total of isolates	26	16	14	4

3.3. PFGE types

Seventeen PFGE types were identified, nine being for *S.* Typhimurium (T1–T9) (T1 includes *S.* 4,[5],12:i:- isolates), two for *S.* Mbandaka (M1, M2) and one for each of the other serotypes (Table 3 and Fig. 1).

3.3.1. S. Typhimurium and S. 4,[5],12:i:-

The T1 PFGE type was identified in 11 pig sample isolates collected in three abattoirs (A, B and C) on different dates. Among these were the three samples of the monophasic variant *S.* 4,[5],12:i:- (Table 3). In abattoir B, type T1 was isolated in two consecutive carcasses (Nos. 17 and 18). The T2 type was identified in two different isolates collected in abattoir A (Nos. 1 and 100) sampled within the period of a year. The T3 type was identified in six isolates collected in two different abattoirs and in abattoir C a positive identification was made on the meat handler who performed the deboning operation of a positive carcass (No. 30). In abattoir D, three consecutive carcasses (Nos. 41, 42 and 43) and two lymph nodes samples from consecutive pigs numbered 45 and 46 tested positive for the T3 and T4 types respectively (Table 3). The Type T4 was identified in two different abattoirs (D and H). The T8 type corresponds to *S.* Typhimurium DT104 and was recovered from six different pig samples and a meat handler in abattoir H. This strain was detected in pig No. 78 (lymph nodes, carcass and meat), carcass and/or meat samples from pigs processed in the same day (Nos. 76 and 77) and the meat handler who performed the deboning operations on these carcasses (Table 3).

3.3.2. S. Derby

A single PFGE type (D1) was identified in the 11 isolates of *S.* Derby collected in four abattoirs (A, B, C and F). In abattoir A this type was detected in the lymph node sample of pig No. 2 and in the subsequent carcass (No. 3). In abattoir C it was possible to identify this type in the three different samples collected from pig No. 35 (Table 3).

3.3.3. S. Rissen

Four isolates of *S.* Rissen were identified, all corresponding to a single PFGE type (R1), originating in three different abattoirs (B, D and G). One of the positive samples was the meat handler who performed the deboning operations of a positive carcass (No. 70) (Table 3).

3.3.4. Other serotypes

Two different types (M1, M2) were identified in the three isolates of *S.* Mbandaka, collected in three abattoirs (D, F and G). Other

Table 3
Distribution of *Salmonella* clones among pig samples and meat handlers recovered in different abattoirs.

Abbatoir	Date	Pig[a]	Ileocecal limph nodes	Carcass	Meat	Meat handler
A	06.08.07	1[1]	*S.* Typh. T2[b]			
A	"	2	*S.* Derby D1			
A	"	3		*S.* Derby D1		
A	24.09.07	7			*S.* Typh. T1	
A	"	8			*S.* 4,[5],12: i :- T1	
A	"	9[2]	*S.* Typh. T1			
A	14.05.08	93[1]	*S.* Typh. T1			
A	20.05.08	96		*S.* Typh. T1		
A	28.08.08	99[2]	*S.* Derby D1			
A	"	100	*S.* Typh. T2			
B	16.10.07	13	*S.* 4,[5],12: i :- T1			
B	30.10.07	17		*S.* Typh. T1		
B	"	18[3]		*S.* Typh. T1		
B	05.11.07	21	*S.* Typh. T1			
B	08.11.07	22		*S.* Derby D1		
B	27.11.07	26	*S.* Rissen R1			
C	10.12.07	29	*S.* 4,[5],12: i :- T1	*S.* Typh. T5		
C	"	30[4]	*S.* Typh. T3		*S.* Typh. T1	*S.* Typh. T3
C	12.12.07	31	*S.* Typh. T5			
C	"	35[4]	*S.* Derby D1	*S.* Derby D1	*S.* Derby D1	
C	18.12.07	36			*S.* Typh. T6	
C	"	37	*S.* Derby D1			
D	21.01.08	41[5]	*S.* Mbandaka M1	*S.* Typh. T3	*S.* Typh.T7	
D	"	42		*S.* Typh. T3	*S.* Typh. T3	
D	"	43[6]		*S.* Typh. T3	*S.* Typh. T7	
D	29.01.08	44[6]		*S.* London L1		
D	"	45	*S.* Typh. T4	*S.* London L1	*S.* Rissen R1	
D	"	46[5]	*S.* Typh. T4			
E	08.02.08	47				*S.* London L1
F	13.02.08	51	*S.* Give G1			
F	15.02.08	55	*S.* Derby D1	*S.* Derby D1		
F	"	56	*S.* Mbandaka M1			
F	18.02.08	57			*S.* Typh. T9	
F	20.02.08	60	*S.* Enteritidis E1			
F	"	61			S. Derby D1	
G	05.03.08	67[7]	*S.* Mbandaka M2			
G	11.03.08	70[7]		*S.* Rissen R1		*S.* Rissen R1
G	28.03.08	75[3]	*S.* Sandiego S1			
H	03.04.08	76			*S.* Typh. T8[c]	
H	04.04.08	77		*S.* Typh. T8[c]	*S.* Typh. T8[c]	*S.* Typh. T8[c]
H	"	78	*S.* Typh. T8[c]	*S.* Typh. T8[c]	*S.* Typh. T8[c]	
H	18.04.08	86[8]	*S.* Give G1			
H	19.04.08	89[8]	*S.* Typh. Ty4			

[a] Superscript numbers [1,2,4,5,6,7,8,] indicate pigs with same farm of origin.
[b] *S.* Typhimurium and S. 4,5,12:i:- corresponds to genotypes T1–T9; *S.* Derby to D1; *S.* Rissen to R1; *S.* Mbandaka to M1,M2; *S.* London to L1; *S.* Give to G1; *S.* Enteritidis to E1; *S.* Sandiego to S1.
[c] Corresponds to *S.* Typhimurium DT104.

serotypes identified corresponded to single profiles (L1, G1, E1 and S1). The L1 PFGE type was identified in two consecutive carcasses (Nos.44 and 45) at abattoir D (Table 3).

In 11.5% (95% CI 3.0–31.3) of the pigs that had *Salmonella*-positive lymph node samples, it was possible to identify the same genotype in other samples (carcass and/or meat) (Nos.35, 55 and 78) (Table 3).

Regarding meat contamination, 28.6% (95%CI 9.6–58.0) of the positive meat samples belonged to *Salmonella*-positive carcasses where the same genotype was also identified (Nos. 35, 42, 77 and 78); 14.3% (95% CI 25.2–43.9) corresponded to *Salmonella*-positive pigs (Nos. 35, 78) where the same genotype was also identified in carcass and lymph node samples. Concerning meat handlers, *Salmonella* was isolated in 9.3% of the samples collected and 75% (95%CI 21.94–98.68) of positive meat handlers were in contact with positive meat samples, where the same genotype was isolated (Table 3).

4. Discussion

In this study we observed a high *Salmonella* prevalence in slaughter swine and carcasses originating from multiple abattoirs supporting previous data (EFSA, 2008a; Vieira-Pinto et al., 2005). Importantly, we were also able to trace the occurrence of *Salmonella* along critical points in the slaughterhouse process. *Salmonella* was isolated in all abattoirs, and, with the exception of one abattoir (E), there were positive results from both lymph nodes and carcasses. It should be noted that despite the random selection of pigs, a common holding origin was used for the sampled animals in the different abattoirs (Table 3). Different *Salmonella* PFGE types were identified in the lymph nodes of these pigs, even when collected during the same time period, suggesting a diverse *Salmonella* population in the environment preceding the slaughterhouse (production level, transport and lairage) (Table 3). Contrary to what would be expected, albeit with marginal significance, abattoirs with the more hygienic scalding system had higher carcass contamination results when compared with those abattoirs using the less hygienic system (Table 1). This is probably due to other subsequent operations (such as polishing and evisceration) as the pig carcasses pass through the flaming device, a contamination decreasing procedure, after scalding but prior to the referred operations. The contamination from the polishing operation can also be due to the equipment, in particular the flails and brushes of the polisher used during polishing, and the carcass splitter following polishing which should both be

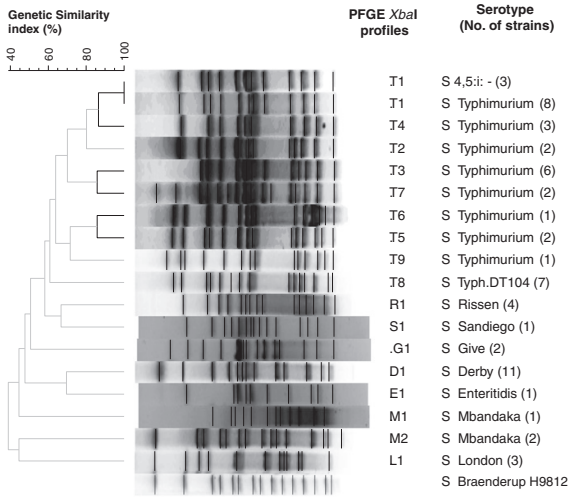

Fig. 1. Seventeen representative *Xba*I PFGE profiles of the 60 *Salmonella* isolates analyzed and their similarity dendogram (*S.* Braenderup H9812 was also restricted with *Xba*I and used as a size standard).

disinfected with water at 82 °C between each carcass, however, the water used is frequently at a lower and ineffective temperature (Botteldoorn et al., 2004; De Busser et al., 2011; Lo Fo Wong et al., 2002; Vieira-Pinto et al., 2005). Abattoirs B and G, in spite of returning positive results from lymph node and carcass tests had no positive meat samples, probably resulting from better hygienic parameters and individual performance in the cutting and deboning rooms. However, in abattoir G, as with H, there is a causal link between the positive meat handler and the positive processed carcasses (Table 3).

This study revealed high rates of occurrence in lymph nodes, similar to previous studies (EFSA, 2008a; Vieira-Pinto et al., 2005). Several authors refer to a correlation between the slaughter of asymptomatic *Salmonella*-carrier pigs and carcass and meat contamination (Berends et al., 1998b; De Busser et al., 2011; Delhalle et al., 2009; Vieira-Pinto et al., 2005, 2006). In our study, this association was only verified in three positive pigs carrying the same PFGE types in the lymph node and subsequent samples (carcass and/or meat) (Table 3). Furthermore, in Portugal there is a strong tradition of pork meat products that include the use of pork bowel in smoked and dried fermented sausages. In the abattoir, after evisceration and Official Veterinary Inspection, offal are washed and prepared in a separate room, including the removal of intestinal lymph nodes. This operation is a potential source of cross-contamination from the high percentage of *Salmonella*-positive, but consistently normal in appearance, lymph nodes. Besides the contribution to the complex cycle of contamination at slaughter house level, the raw bowel enters into the food chain through dispatch to butcher shops or meat plants where it is processed again, assuming a further role in *Salmonella* dissemination.

The level of carcass contamination was above the EU baseline survey value and points to a hygiene problem, as EU food law imposes the surveillance of *Salmonella* in pig carcasses under criteria for process hygiene (Anonymous, 2005). In several consecutive carcasses, obtained from pigs which tested negative at lymph node level, the same PFGE type was identified, suggesting that the source of contamination was common and possibly environmental (such as from contact with equipment). This pattern was particularly frequent in abattoir D, with a high rate of pigs slaughtered per hour. In another case, a positive carcass was related to a positive lymph node sample from a previous animal, which contaminated contiguous carcasses in the slaughter line and then propagated the contamination to the cutting and deboning room, including the meat handler and several meat samples (Table 3).

Fresh meat is not regularly tested for *Salmonella* contamination at meat plants as is required of minced meat or prepared meat by EU legislation (Anonymous, 2005). In this study the observed contamination of meat is much higher than the most recent published Portuguese data (EFSA, 2011) but nevertheless lower than other authors report (Delhalle et al., 2009). Moreover, strong evidence of cross-contamination between carcasses and meat is present, supporting the hypothesis that contamination is transferred between successive phases of pig slaughter and processing, as other authors have referred to (Berends et al., 1998b; Botteldoorn et al., 2004; De Busser et al., 2011; Lo Fo Wong et al., 2002; Swanenburg et al., 2001; Vieira-Pinto et al., 2005, 2006).

In three of the *Salmonella*-positive meat handlers, the PFGE type coincided with that found on the carcasses and/or manipulated meat samples, as they were responsible for the cutting and deboning operations of the respective carcass (Table 3). This occurrence identifies a cross-contamination risk point and, again, a hygiene and public health problem, as besides the possibility of infection, workers could act as a vehicle for *Salmonella* transmission to the community. A previous study concerning the topic of cross contamination, carried out with the participation of meat handlers from these abattoirs, revealed that half of the respondents did not seem to be aware of the importance of changing clothes and working instruments, when they move from the tasks executed in 'dirty spaces' (located at the abattoir) to 'clean spaces' (deboning room) in the same meat plant (Gomes-Neves et al., 2011). Although it is generally accepted that the hands of food handlers are an important vehicle for food cross contamination, the same study also observed that a high proportion of respondents from the group of meat handlers did not know all the required steps as part of a correct hand washing procedure. There was also a general lack of knowledge of microbiological food hazards, i.e. *E. coli*, *Salmonella*, *Campylobacter* and *L. monocytogenes*, and the related risks to their own and public health (Gomes-Neves et al., 2011).

Our results regarding serotypes and their respective proportions are consistent with the Baseline EU survey and other studies of slaughtered pigs, where *S.* Typhimurium is the most frequent in all groups of samples, followed by *S.* Derby, *S.* Rissen and *S.* 4,[5],12:i:- (EFSA, 2008a, 2008b). The largest variation was noticed in the lymph nodes, where all of the serotypes were isolated except *S.* London (Table 2), which was not previously reported in Portuguese data. In the carcass and meat samples only four serotypes were isolated and the most prevalent were *S.* Typhimurium and *S.* Derby (Table 2), which is compatible with the EU survey results on carcass contamination, although Portugal was not included in that part of the study (EFSA, 2008a).

In this study, different *Salmonella* PFGE types could be identified in samples from the same pig, in accordance with the results of other authors and unveiling cross-contamination critical points (Botteldoorn et al., 2004; De Busser et al., 2011). This finding contrasts another study, where it was shown that the same *Salmonella* genotype was identified in all positive samples from the same pig, with only one exception (Vieira-Pinto et al., 2006).

Although there is causal linkage between *Salmonella* contamination in carcasses and lymph nodes, this path of transmission is seen to be less relevant for this study when compared with other previous studies, underlining the importance of spreading contamination across carcasses, meat and meat handlers' hands, unveiling an important public health problem (Berends et al., 1998b; Botteldoorn et al., 2004; Vieira-Pinto et al., 2005, 2006). As other authors have previously noted, the main contamination source is probably a continuous contamination cycle between slaughtered pigs, the environment and the carcasses (Botteldoorn et al., 2004; De Busser et al., 2011; Swanenburg et al., 2001).

Additionally, the dominant serotypes identified in this study are commonly associated with human disease (EFSA, 2011; King et al.,

2011), including the monophasic variant *S.* 4,[5],12:i:- (5%) considered emergent in humans (Dionisi, et al., 2009; Lucarelli et al., 2010; Hauser et al., 2010; Switt et al., 2009) and *S.* Typhimurium DT104 (12%) (Antunes et al., 2006). Of particular concern is the presence of this strain on a meat handler, underlining the importance of the abattoir environment in spreading human-health threatening clones.

In spite of the fact that the significance of the present results is limited in part by the sample size, this study indicates that pork meat is an important source of *Salmonella* and that abattoir procedures could promote its contamination. Although swine can harbor *Salmonella* before slaughter, the abattoir environment can contribute to further cross-contamination along the slaughter line, including contact with meat handlers. There has been a lack of recent data in Portugal concerning contamination of pork products by *Salmonella* and further studies should clarify and quantify this transference of contamination. The primary production phase and the slaughterhouse environment have been found to be the main sources of the contamination of carcasses (Berends et al., 1998a,1998b; Botteldoorn et al., 2004; De Busser et al., 2011; Delhalle et al., 2009; Vieira-Pinto et al., 2005, 2006) and, in order to improve standards in the post-harvest pork meat chain, measures have to be taken at these stages. Reducing the prevalence of *Salmonella* positive pigs at the primary production phase can significantly decrease one of the main sources of contamination at the abattoir (De Busser et al., 2011). However, the need to invest in general hygiene improvement, meat handlers' training, good manufacturing practice and HACCP implementation (Delhalle et al., 2009; Gomes-Neves et al., 2011; Lo Fo Wong et al., 2002), remains crucial to reducing cross-contamination and to maintain the level of contamination as low as possible in pork meat.

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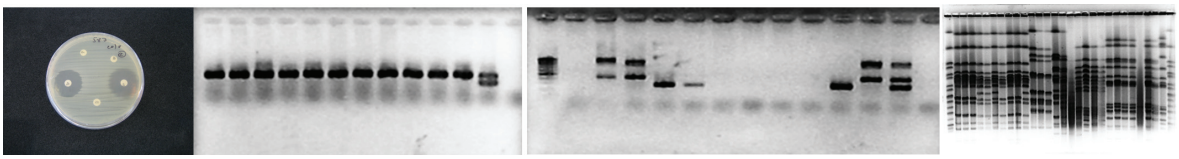
Chapter 2. One health: Antimicrobial Resistance entering the food chain.

Paper II. Clinically relevant multidrug resistant *Salmonella enterica* in swine, at slaughter: filling the gaps in the food chain.



Clinically relevant multidrug resistant *Salmonella enterica* in swine, at slaughter: filling the gaps in the food chain

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Abstract

The presence of clinically relevant *Salmonella* serotypes in slaughtered swine, carcasses, meat and meat handlers is scarcely studied. In this work, we characterized resistance phenotypes and genotypes in 60 *Salmonella* isolates from swine (lymph nodes, carcasses, meat) and meat handlers of Portuguese abattoirs (July 2007-August 2008). More than 50% of the isolates were resistant to tetracycline (T) [70%, *tet*(A)/*tet*(B)/*tet*(G)], streptomycin (S) [63%, *aadA2/strA/strB*], sulfamethoxazole (Sul) [62%, *sul1/sul2/sul3*] and ampicillin (A) [57%, bla_{PSE-1}/bla_{TEM}] and 37% carried class 1 integrons. Multidrug resistance was frequently observed in isolates (63%; n=38/60) from all samples and most of serotypes, including the ones frequently observed in human infections [*S*. Typhimurium (78%), *S*. 4,[5],12:i:- (75%), *S*. Derby (55%), *S*. Rissen (75%), *S*. London (75%)]. The *S*. 4,[5],12:i:- isolates mostly presented ASSuT phenotype [bla_{TEM}/*strA-strB/sul2/tet*(B)], typical of the European clone, being here firstly described with a ST phenotype [*strA-strB-tet*(A)-*tet*(B)]. Multidrug resistance [ANSSuT; bla_{TEM}-*strA-strB-sul2-tet*(A)] in *S*. London was also firstly reported. The identification in slaughter swine and meat handlers' samples of *Salmonella* serotypes carrying antibiotic resistance features similar to the previously characterized in clinically isolates provides the lacking food-chain evidences to link their transmission from animals to humans. The abattoir environment and the slaughter operations seem not only to maintain MDR serotypes originated from the pig reservoir, but also propagate them through cross-contamination processes, involving meat handlers.

Keywords: *Salmonella*, antimicrobial agents, swine, pork meat, *S*. Typhimurium monophasic variant

1. Introduction

Ten to twenty percent of human *Salmonella enterica* infections in the EU may be attributable to pig sources, as reported by EFSA in 2010 [1]. In addition, an increasing antibiotic resistance trend has been consistently observed in pigs and pork products [2], which could reach humans through the food chain [1]. Particular multidrug-resistant (MDR) *S. enterica* isolates with clinical relevance, such as *S*. Typhimurium monophasic variant (*S*. 4,[5],12:i:-), *S*. Typhimurium DT104 and *S*. Rissen have been described in piggeries or in pork [3,4,5,6]. In spite of the evidence that the animal setting is a worldwide reservoir of MDR strains [2,5], their characterization in swine at slaughter, as well as in meat handlers' samples, has been scarcely studied, a critical point to link swine as a source of human *Salmonella* infection cases [1,2,3]. Thus, in this work, we assessed the presence of clinically relevant multidrug resistant *Salmonella* serotypes in slaughtered swine, carcasses, meat and meat handlers from 8 abattoirs in Portugal.

2. Material and Methods

2.1. *Salmonella* isolates, serotypes and PFGE types

A total of 60 *S. enterica* isolates (56 from swine: 26 ileoceacal limph nodes samples, 16 carcass swabs and 14 meat samples; 4 from meat handlers' hands) collected between July 2007 and August 2008 from 8 abattoirs (A-H) (Table 1) were included in this study [7]. Briefly, isolates belonged to nine serotypes, which included 32 *S*. Typhimurium and 3 *S*. 4,[5],12:i:-, 11 *S*. Derby, 4 *S*. Rissen, 3 *S*. London, 3 *S*. Mbandaka, 2 *S*. Give, 1 *S*. Enteritidis and 1 *S*. Sandiego. After PFGE analysis those with a SD (Dice Band-based similarity coefficient) value > 85% were considered to belong to the same PFGE-type. As shown in Table 1, the *S. enterica* serotypes belonged to 17 PFGE types, nine being *S*. Typhimurium (T1-T9) (T1 includes S. 4,[5],12:i:- isolates), two *S*. Mbandaka (M1, M2) and one of each of the other 6 serotypes.

Table 1. PFGE types, antimicrobial resistance profiles and class 1 integrons of Salmonella serotypes isolated from swine and meat handlers

Serotype[a] / PFGE type[b] (number of isolates)	Abattoir[c] (number of isolates)	Sampled material (number of isolates)	Resistance phenotype[d]/ Resistance genes profile	Class 1 integrons (gene cassettes) (number of isolates)
Typhimurium/T1 (8)	A (4); B (3); C (1)	Lymph node (3); Carcass (3); Meat (2)	(AMP), (STR), (SUL), TET, (TMP)/ (bla_{TEM}), (*strA-strB*), (*sul2-sul3*), (*tet*(A)-*tet*(B)),	-
4,[5],12:i:-/T1 (3)	A (1); B (1); C (1)	Meat (1); Lymph node (2)	(AMP), STR, (SUL), TET/ (bla_{TEM}), *strA-strB*, (*sul2*), (*tet*(A)), *tet*(B)	-
Typhimurium/T2 (2)	A (2)	Lymph node (2)	-	600 (1)
Typhimurium/T3 (6)	C (2); D (4)	Lymph node (1); Carcass (3); Meat (1); Meat handler (1)	AMP, (STR), (SUL), TET/ bla_{TEM}, (*sul3*), (*tet*(A)), (*tet*(B))	400 (3)
Typhimurium/T4 (3)	D (2); H (1)	Lymph node	AMP, STR, SUL, TET/ bla_{TEM}, (*strA-strB*), *sul3, tet*(B), (*tet*(G))	-
Typhimurium/T5 (2)	C (2)	Lymph node (1); Carcass (1)	(AMP, TET)/ (bla_{TEM}, *tet*(A), *tet*(B))	600 (1)
Typhimurium/T6 (1)	C	Meat	AMP, CHL, STR, SUL, TMP/ bla_{TEM}, *cmlA1*, *sul2*	-
Typhimurium/T7 (2)	D (2)	Meat	AMP, STR, SUL, TET / bla_{TEM}, *sul3, tet*(B)	-
Typhimurium DT104/T8 (7)	H (7)	Lymph node (1); Carcass (2); Meat (3); Meat handler (1)	AMP, CHL, STR, SUL, TET / *bla*PSE-1, *floR, aadA2, sul1*, (*tet*(A)-*tet*(G))	1000 (*aadA2*) + 1200 (*bla*PSE-1) (7)
Typhimurium/T9 (1)	F	Meat	-	-
Derby/D1 (11)	A(3); B(1); C(4); F(3)	Lymph node (5); Carcass (4); Meat (2)	(STR, SUL, TET) / (*aadA2, sul1, tet*(A))[e]	1000 (*aadA2*) (6)
Rissen/R1 (4)	B (1); D (1); G (2)	Lymph node (1); Carcass (1); Meat (1); Meat handler (1)	(AMP), (CHL), (STR), (SUL), TET, (TMP)/ (*bla*TEM), (*cmlA1*), (*aadA2*), (*sul1-sul3*), *tet*(A), (*dfrA*12)	1000 (1); 2000 (*dfrA12, orfF*, *aadA2*)(2)
Mbandaka/M1 (1)	G	Lymph node	STR/*strA-strB*	-
Mbandaka/M2 (2)	D (1); F (1)	Lymph node	-	-
London/L1 (3)	D (2); E (1)	Carcass (2); Meat handler (1)	(AMP, NAL, STR, SUL, TET) / (bla_{TEM}, *strA-strB*, *sul2, tet*(A))[f]	800 (1)
Give/G1 (2)	F (1); H (1)	Lymph node	-	-
Enteritidis/E1 (1)	F	Lymph node	NAL	-
Sandiego/S1 (1)	G	Lymph node	-	-

[a] *PCR assays for the identification of Salmonella enterica serotype Typhimurium DT104/U302 [4] and Salmonella enterica 4,[5],12:i:- were performed [6].*

[b] *Clones are indicated by capital letters and a subindex (Typhimurium and S.4,[5],12:i:-, Ty1-Ty9; Mbandaka, M1-M2; Derby, D1; Rissen, R1; London, L1; Give, G1; Enteritidis, E1; Sandiego, S1)*

[c] *Abattoirs are identified from A to H;*

[d] *AMP, ampicilin; CHL, chloramphenicol, NAL, nalidixic acid; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; Variable presence of a given resistance phenotype/ genotype among isolates belonging to the same PFGE-type appears between parenthesis; (-) Absence of antibiotic resistance.*

[e] *This resistance phenotype and genotype is present in 6 isolates out of 11.*

[f] *This resistance phenotype and genotype is present in 2 isolates out of 3.*

2.2. Antimicrobial susceptibility testing

All *S. enterica* isolates were tested for antimicrobial susceptibility by the disk diffusion method, following CLSI standards [8]. Ten antimicrobial agents were tested: ampicillin (A), gentamicin (G), kanamycin (K), streptomycin (S), ciprofloxacin (Ci), nalidixic acid (N), chloramphenicol (C), tetracycline (T), sulfamethoxazole (Su) and trimethoprim (W). *Escherichia coli* ATCC 25922 was the control strain. Ampicillin resistant isolates were further tested for susceptibility to extended-spectrum β-lactams (ceftazidime, ceftriaxone, cefotaxime, cefepime, cefoxitin, aztreonam and imipenem) and the double disk synergy test for ESBL detection was also performed [8]. An isolate was scored MDR if it had reduced susceptibility to three or more structurally unrelated antibiotics.

2.3. Characterization of antimicrobial resistance genes and class 1 integrons

Genes coding for resistance to ampicillin (bla_{TEM}, bla_{PSE-1}, bla_{OXA-30}), streptomycin (*aadA, strA-strB*), tetracycline [*tet*(A), *tet*(B), *tet*(G)], sulfamethoxazole (*sul1, sul2, sul3*), chloramphenicol (*floR, cmlA, catA*) and trimethoprim (*dfrA1, dfrA12*) were searched by PCR, using primers and conditions previously described [4,6]. The detection and characterization of class 1 integrons was also performed by PCR and sequencing as reported [4]. Positive and negative and controls were included in all PCR assays.

3. Results and discussion

3.1. Percentage of antibiotic resistance and resistance phenotypes

Antibiotic resistance was found in 75% of all *Salmonella* isolates, 63% of which were MDR. Interestingly, lowest rates of resistance were observed in isolates from lymph nodes (58%) comparing to isolates from samples of subsequent slaughter operations (94% from carcasses and 86% from meat) or meat handlers (75%). These results suggest that the abattoir environment and the slaughter operations are contributing to the spread of antibiotic resistant *Salmonella*.

Resistance to tetracycline (n=42; 70%), streptomycin (n=38; 63%), sulfamethoxazole (n=37; 62%) and ampicillin (n=34; 57%) was detected in a higher frequency than chloramphenicol (n=9; 15%), trimethoprim (n=5; 8%) and nalidixic acid (n=3; 5%), which may reflect the high usage of the first antibiotics in food-producing animals [9]. These resistance phenotypes were also the most frequently reported in *Salmonella* isolates from humans, pigs and pork meat in EU [2], in spite that overall in lower percentages than in our study. Susceptibility to extended-spectrum β-lactams and absence of ESBL-producing strains was found. The ASSuT (38%), ACSSuT (16%) and SSuT (13%) were the most

frequent resistance phenotypes among our isolates, which may provide a selective advantage in the intensive animal production setting.

3.2. Dissemination of resistance determinants and serotypes

A diversity of resistance genes encoding resistance to different families of antibiotics was detected among isolates of different serotypes/clones and spread in different pig abattoirs and samples, as shown in Table 1. Thirty-seven percent of the isolates (n=22/60) were positive for class 1 integrons (400-2000 bp), namely *S*. Typhimurium (12/22), including all DT104 isolates 123 (7), *S.* Derby (6/22), *S.* Rissen (3/22) and *S.* London (1/22), a similar rate to the observed in *Salmonella* from pork food products collected in Portugal [4]. Moreover, these strains shared identical resistance determinants with previously national and international widespread serotypes/clones from human, pork food products, food producing animals and, particularly, swine, supporting their involvement in human infections [3,4,6,10,11,12].

S. Typhimurium (9 PFGE-types) identified in swine samples (6 abattoirs) and in meat handlers (2 abattoirs) presented high antimicrobial resistance (88%, n=28/32) and was mostly MDR (78%, n=25/32), supporting the trend described in other works for this serotype from other sources/niches [2,4,12,13]. The phenotype ASSuT was the most prevalent (47%, n=15/32) in this serotype, with some PFGE-types identified in diverse samples in the same abattoir, as a result of cross-contamination, and/or disseminated in different abattoirs (Table 1). Particularly, *S.* Typhimurium DT104 (T8) isolated in lymph nodes, carcasses, meat and a meat handler in a single abattoir, clearly related to cross-contamination originated from lymph node samples, presented the typical MDR pattern, previously identified in food, human and piggeries isolates [4,6], suggesting that slaughter operations play a role in the spread of such strains. *S.* 4,[5],12:i:- resistant to different antibiotics isolated from lymph nodes and in this study, firstly, in Portugal, in meat samples in 3 different abattoirs, indicates that pork food products is an important vehicle of this emergent serotype. These isolates presented 100% of clonal relatedness with *S*. Typhimurium (T1) recovered from the same abattoirs and were mostly associated with the R-type ASSuT [*bla*$_{TEM}$/*strA-strB*/*sul2/tet*(B)], typical of the “European clone” [3,5,6,13] of monophasic strains and previously characterized in Portuguese piggeries [6]. This data suggests that resistance determinants of both *S.* Typhimurium and its monophasic variant were maintained in the pig reservoir in spite of their genetic evolution [14]. Additionally, one 148 of the *S*. 4,[5],12:i:- isolates presented the R-type ST [*strA-strB/tet*(A)/*tet*(B)], to our knowledge here, firstly, reported, which may be considered in the surveillance of this emergent serotype.

S. Rissen, other emergent serotype, revealed common resistance features [tetracycline-*tet*(A) and/or typical class 1 integron-*dfrA12-orfF-aadA2*] of this serotype [4,6,11].

The presence of these isolates in human, food products and piggeries [4,6] and in samples collected in this study, indicates its dissemination throughout the food chain. Additionally, this study shows that meat handler's contamination could provide an alternative route for community spread of this increasingly observed MDR serotype. Most isolates of the *S*. Derby, a serotype frequently isolated in swine [15], identified in this study in lymph nodes, carcasses and meat from different abattoirs, presented R-type SSuT [*aadA2, sul1, tet*(A)] , including a class 1 integron (*aadA2*). These resistance features have been associated with isolates of human, food products and food producing animals of this serotype [4,10,12]. Interestingly, *S*. London, a scarcely reported serotype [15], revealed the ANSSuT pattern [(bla_{TEM}/*strA-strB/sul2/tet*(A)] dispersed in two abattoirs. These findings alerts for the possible emergence of a new MDR serotype. *S.* Mbandaka and *S*. Enteritidis, rarely identified in swine production [11,15], revealed only resistance to streptomycin and nalidixic acid, respectively, disseminated in different abattoirs.

In conclusion, this study revealed a high frequency of multidrug resistance in different *Salmonella* serotypes from slaughtered swine, carcasses, meat and meat handlers, including the ones frequently observed in human infections. The European MDR clone of *S*. Typhimurium monophasic variant in swine at abattoir level in Portugal and MDR in *S*. London were firstly reported in this study. The abattoir environment and the slaughter operations seem not only to maintain MDR serotypes originated from the swine 173 reservoir, but also propagate them through cross contamination processes, involving meat handlers. The enlightenment of the linking between swine and human salmonellosis throughout the food chain is of interest for epidemiological, animal health and public health purposes.

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Chapter 3. The weakest link: Meat handlers - from food producing animals to public health

Paper III. Meat handlers training in Portugal: a survey on Knowledge and Practice.

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Meat handlers training in Portugal: A survey on knowledge and practice

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ABSTRACT

Professional training for meat handlers is an European Community food law requirement in order to apply HACCP principles and achieve food safety goals. A self-administered questionnaire designed to assess "Knowledge" and "Practice" of public hygiene measures was completed by meat handlers (MH) ($n = 159$) in slaughterhouses in Portugal. A significant proportion of the group (72.7%) has had professional training in two different areas: Good Practice in Food Industry (12.03%) and Work Safety and Hygiene (22.8%); 37.9% of the respondents have had training in both areas. However 24.5% of the subjects have never had training. Meat handlers with professional training in Good Practice in Food Industry (GPFI) and in both areas (BT) have had the highest proportions of correct answers in Knowledge (66.92 ± 16.36 and 67.26 ± 21.05, respectively) and Practice questions (70.53 ± 17.47 and 68.67 ± 22.58, respectively).

The results of this study point to the need to improve training, particularly in Good Practice in Food Industry, thus enabling meat handlers to achieve more correct answers in Knowledge and Practice. The development of evaluation criteria for the effectiveness of professional training is crucial to protect Public Health.

1. Introduction

The increasing incidence of food borne diseases has been assigned to many different factors, including population growth, changes in food preparation habits, a rise in the number of food-service establishments, increased consumption of food outside the home and a lack of food safety training and education among consumers and food handlers (Motarjemi & Käferstein, 1999). Worker mishandling of food is one of the major causes of food borne disease outbreaks (WHO, 2000). Because outbreaks often lead to severe economic losses, food handler training is an important business strategy for managing food safety risks. Moreover, food handler training is seen as one strategy by which food safety can be increased, offering long-term benefits for the food industry (Smith, 1994). In addition, the European Parliament has adopted in April 2004 the Regulation (EU) No. 852/2004, underlining the need for all the food businesses to identify the steps of the production process in order to ensure food safety and this has been applied to all EU food businesses since the 1st January 2006. The main change relates to food safety management systems, i.e. risk-based methodologies to ensure food safety. The law's implementation recognises education of food handlers as a crucial line of defence in the prevention of food borne illnesses (Legnani, Leoni, Berveglieri, Mirolo, & Alvaro, 2004; Martínez-Tomé, Vera, & Murcia, 2000; Sun & Ockerman, 2005; Worsfold, 2001). Food business operators shall ensure that all stages of production, processing and distribution of food under their control satisfy the relevant hygiene requirements laid down in the Regulation (EU) No. 852/2004 (Jevšnik, Hlebec, & Raspor, 2008). A successful implementation of the procedures based on the HACCP (Hazard Analysis and Critical Control Points) principles will require the full cooperation and commitment of food business employees and to this end they should undergo training. Under the personal program of HACCP, employees must be trained in such areas as food safety, manufacturing controls and personnel hygiene. Once HACCP plans have been established, employees must be trained to manage any critical control points (CCPs). The necessity of application of the HACCP principles introduced by the *Codex Alimentarius* 30 years ago became law in Portugal in 1998 (Diário da República, 1998), and the Portuguese law has recently established the requisites for a "handler card" (Diário da República, 2006) for meat handlers (MH) working in meat retail businesses, to apply from 1st August 2008. To obtain this

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card, it is necessary to attend 15 h of mandatory training on the following subjects: Meat Hygiene, Food Microbiology, Handlers' Personal Hygiene, Working spaces and Equipments' Hygiene, Packaging of meat and meat products, Hygiene of meat selling and delivery, Food Safety and HACCP, Work Safety and Hygiene. However, this training and this card are not required for working in abattoirs and deboning rooms, where it is considered that the EU regulations No. 852/2004 and No. 853/2004 regulate the need for professional training. The Portuguese general law that regulates work conditions has a legal requirement of 35 h of yearly training for all workers (Diário da República, 2003, 2004). Recently, much has been written specifically on training in the food industry, but a great part of it is rather specific in nature and has been limited to discussions on single segments, primarily hotels and restaurants (Barrows, 2000; Seaman & Eves, 2006). There is a general lack of information about professional training for slaughterhouses and deboning rooms' workers.

The aim of this study was to evaluate and compare the level of general knowledge and practice of meat handlers from slaughterhouses and meat plants from northern Portugal, evaluating the professional training they have received. To our knowledge, this is the first survey on meat handling knowledge and practice in Portugal. Other similar studies have been reported in several countries focusing on food handlers (Gomes-Neves, Araújo, Ramos, & Cardoso, 2007; Jevšnik et al., 2008; Nel, Lues, Buys, & Venter, 2004; Seaman & Eves, 2006; Walker, Pritchard, & Forsythe, 2003).

2. Material and methods

2.1. Questionnaire design

The self-administered questionnaire used in this study comprises 24 multiple choice questions with three or four possible answers, including "do not know" for the purpose of minimizing the possibility of selecting the correct answer by chance. In addition, the questionnaire has seven questions related to demographic and job characteristics of the respondents (age, gender, number of years of formal education, age at the beginning of professional activity, job description and years of experience in the present activity and present company, professional training and the opinion to additional training). The present questionnaire has been adapted from a questionnaire used in a previous study (Gomes-Neves et al., 2007).

The questions were designed and structured in two groups. A group of questions designated "Knowledge" (14 questions) was intended to assess the respondent's knowledge about HACCP, microbiologic hazards development, food poisoning and food borne illness, safety and health requirements, high-risk food groups, dirty and clean areas in the workspace and water temperature in knife sterilisers. A second group of questions designated "Practice" (10 questions) was designed to assess respondents' habits focused on personal hygiene practice and cross contamination, working surfaces and instrument washing requirements and products, meat and chopped meat storage temperatures, freezing temperatures, temperature ranges and food poisoning agents development, water treatment and non-potable water use, as water supply and quality and food security and safety are intertwined (Kirby, Bartram, & Carr, 2003; Table 1).

The participants answering the questionnaire have remained anonymous. Each participant has been informed of the purpose of the survey and that confidentiality would be assured.

2.2. Questionnaire delivery

The questionnaire has been delivered in person in seven red meat abattoirs with deboning rooms, during routine meat inspection of the Veterinary Official Services between May 2007 and May 2008, in two different regions of northern Portugal. In each meat plant, questionnaires have been delivered to all the employees performing tasks related with meat handling. The completed questionnaires have been collected in person one month later.

Table 1
Summary of the focus of the questionnaire contents.

Questions "Knowledge"
HACCP – what is it?
Identify sterile food
What happens to bacteria at 37 °C?
Food borne illness most frequent symptoms
Food borne illness agents transmission
Visual, olfactory or taste checks identify bacteria contaminated meat?
Meat handler hygiene and health and food borne illness agents
Health conditions that are not acceptable in food handling
Potential health consequences of animal intestinal bacteria (*E. coli*, *salmonella*, *Campylobacter* and *Yersinia*)
Listeria monocitogenes and food borne illness
Dirty and Clean workspaces in the abattoir
Food borne agents inactivation
Temperature of knives sterilisers
Questions "Practice"
Working surfaces and instruments washing requirements and products
Potable water use/water supply
Red meat storage temperatures
Chopped meat storage temperatures
Freezing temperatures for meat
Temperature ranges and food poisoning agents development
Different situations that imply hand washing before food handling
Different steps to correct hand wash
Cross contamination and change of working instruments and clothes

2.3. Statistical analysis

The analysis of the questionnaires has been performed using the computer software SPSS® (SPSS Inc., Chicago, IL; version17.0). The significance of the statistical differences of the proportion of correct answers between the groups of participants classified according to professional training has been identified using the Chi-Square test. The 95% confidence intervals (95% CI) of the proportion of correct answers in each group have been estimated according to the Wilson procedure with a correction for continuity (Newcombe, 1998; Wilson, 1927). The differences in the mean scores of Knowledge and Practice questions between the same groups referred to above have been determined using one-way ANOVA with a post-hoc test. In all tests, the statistical significance was two-sided and considered significant at $p < 0.05$.

3. Results

3.1. Quantitative results

3.1.1. Participants' response

Answers have been obtained from all the meat plants contacted, but 10% of the employees have not returned the questionnaire. The number of participants was 159 (115 male and 44 female). All but one were Portuguese. The participants' general characteristics are presented in Table 2.

3.1.2. Comparative analysis of training areas and periods of time among participants

Two different areas of professional training among meat handlers (MH) have been identified: 1. Good practice in food industry (GPFI), and 2. Work Safety and Hygiene (WSH). The vast majority of the respondents (72.7%) has had professional training.

Table 2
Demographic data and job information of the participants.

Participants ($N = 159$)	Average ±SD	Minimum–maximum
Age ($N = 155$)	35.19 ± 10.15	16–58
Years of formal education ($N = 151$)	6.50 ± 2.59	0–13
Age at the beginning of the professional activity ($N = 153$)	15.68 ± 2.53	9–24
Years of experience in the same activity ($N = 133$)	12.65 ± 9.35	0–35
Years in the present company ($N = 154$)	8.89 ± 7.57	0–33

Twelve percent (12.03%) of the respondents have had training in GPFI (12.03%), 22.8% in WSH and 37.9% in both areas (BT). During the previous year, 37.7% of the MH have received between 20 and 35 h of training, but 24.7% have never attended professional training (NT). Eighteen percent have had more than 35 h of training. For comparison purposes, respondents were divided in four professional training groups: GPFI, WSH, BT (both training) and NT (no training). Fifty percent (50.3%) of MH with professional training think that training provides useful information to their work and 64.9% are interested in future training and consider it very important.

3.1.3. Comparative analysis of response to "Knowledge" and "Practice" questions

The group of respondents that has had training in the two areas (BT) reached the highest mean score of proportion of correct answers in the group "Knowledge" (67.26 ± 21.05), followed by the GPFI with a mean score of 66.92 ± 16.36 correct answers; WSH had 49.21 ± 22.77 and NT 47.89 ± 22.63.

In the group of questions "Practice", GPFI has had the highest proportion of correct answers with a mean score of 70.53 ± 17.47, followed by BT (68.67 ± 22.58). The mean score of correct answers for WSH has been of 58.33 ± 19.93, and for NT 63.44 ± 21.70. The difference between the proportion of correct answers to the questions "Knowledge" and "Practice" is statistically significant between the groups (one-way ANOVA Table 3). For the group of questions "Knowledge", a post-hoc test (Tukey HSD test) has defined two different homogenous groups, one with the respondents that have attended GPFI or both areas of professional training and the other with the respondents that have had WSH or no training. In the group of questions "Practice", the same test has assumed two different groups, GPFI and NT. The other two groups (WSH and BT) could not be discriminated. This analysis underlines the fact that, for the questionnaire content and for the purpose of food safety improvement, WSH professional training has no positive impact.

Table 3
Percentage of correct answers to the "Knowledge" and "Practice" questions within each group defined by professional training.

Participant group	Question group	
	Knowledge	Practice
	$N = 14$ questions	$N = 10$ questions
GPFI ($N = 36$)	66.92 ± 16.36[a]	70.53 ± 17.47
WSH ($N = 19$)	49.21 ± 22.77	58.33 ± 19.93
BT ($N = 60$)	67.26 ± 21.05	68.67 ± 22.58
NT ($N = 39$)	47.89 ± 22.63	63.44 ± 21.70
one-way ANOVA	d.f. = 3 $F = 10.393$ $p = 0.000$	d.f. = 3 $F = 3.986$ $p = 0.009$

[a] Mean±1SD.

3.2. Qualitative results

It has been considered important to detect finer differences among the answers to questions that tested the quality of the information sought (Tables 4A and 4B).

3.2.1. "Knowledge" questions (Table 4A)

3.2.1.1. HACCP. Regarding HACCP, 29.3% of MH have never heard of the term and 7% are acquainted with the expression but do not know the meaning of it. Regarding training, from the WSH group, 55.6% answered "do not know" to the question "What is HACCP?" and that proportion increases to 66.7% in the NT group. The proportion of respondents who have given correct answers has been of 63.2% in the GPFI group and 51.7% in the BT group. This group has also had the highest proportion of incorrect answers: 31.7% (NT: 15.4%, WSH: 22.2% and GPFS:15,8%). These differences were statistically significant ($p = 0.000$ using Pearson Chi-Square test).

3.2.1.2. Food poisoning and food borne illness. Almost the half (47.4%) of GPFI, 58.3% of WSH, 53.3% of BT and 43.6% of NT believe that they can identify whether meat is contaminated with food poisoning bacteria by visual, olfactory or taste checks ($p = 0.368$, using Pearson Chi-Square test). Similar results have been obtained in other surveys among food handlers (Gomes-Neves et al., 2007; Jevšnik et al., 2008; Walker et al., 2003) The majority of the MH (60.1%) are aware that insects, other food handlers and raw food are sources of bacteria, but 26.3% of GPFI, 44.4% of WSH, 15% of BT and 33.3% think that MH can only contaminate meat if they are ill ($p = 0.001$, using Pearson Chi-Square test). Twenty six percent (26.3%) of GPFI, 30.6% of WSH, 11.7% of BT and 41.0% of NT believe that MH can only get sick if they have contact with animal blood during work activity ($p = 0.000$, using Pearson Chi-Square test). A significant majority of MH knows that diarrhoea is the symptom that is most associated with food borne illness (85.3%) but 33.3% of NT, 30.6% of WSH and 11.7% of BT have not been able to identify consequences of intestinal bacterial infection (*E. coli*, *Salmonella*, *Campylobacter* and *Yersinia*). These differences among groups of respondents were statistically significant ($p = 0.001$, using Pearson Chi-Square test). Sixty two percent (61.5%) of NT have answered "do not know" to the question that relates *Listeria monocytogenes* with food borne Illness and 55.6% of WSH, 38.3% of BT and 26.3% of GPFI have given the same answer. Sixteen (16.0%) percent of all MH knew the name of the bacteria but did not identify the disease or transmission paths ($p = 0.108$, using Pearson Chi-Square test).

3.2.1.3. Temperature and food poisoning agent's inactivation. Twenty percent of WSH (19.5) and NT (20.4) have answered "do not know" to the question "What happens to bacteria at 37 °C?". More than a half (52.6%) of GPFI, 41.7% of WSH, 51.7% of BT and 28.2% of NT think that pasteurised milk is a sterile product. Among the NT group, 43.6% have not answered the question "identify a sterile food product" ($p = 0.105$, using Pearson Chi-Square test). High temperature has been recognised as a safe method to destroy bacteria by 52.6% of GPFI, 50.0% of WSH, 56.7% of BT and 48.7% of NT but 24.4% of MH think that refrigeration also kills bacteria. The majority (64.6%) of MH knows that 82 °C is the correct temperature for the water in sterilisers for knives and steels in stations located along the slaughter floors (Eustace et al., 2007), but 21.1% of GPFI, 38.9% of WSH, 30.0% of BT and 28.2% of NT have answered incorrectly. The differences between the groups of respondents were not statistically significant.

3.2.1.4. Safety and health requirements. Many MH did not seem to be aware of basic safety and health requirements to work with food. A majority of GPFI, WSH and NT (52.6%, 52.8% and 51.3%, respectively) have not identified skin disease, gastrointestinal disturbances, eye/

Table 4A
Percentage of correct answers and 95% Confidence Intervals[a](CI) of the questions "Knowledge" (qualitative results).

Questions "Knowledge"	% of Correct Answers (95% CI)			
	GPF	WSH	BT	NT
	N=36I	*N* = 19	*N* = 60	*N* = 39
What is HACCP?	63.2 (38.6–82.8)	22.2 (10.7–39.6)	51.7 (38.5–64.6)	17.9 (8.1–34.1)
Identify sterile food	21.1 (7,0–46.1)	25.0 (12.7–42.5)	31.7 (20.6–45.1)	28.2 (15.6–45.1)
What happens to bacteria at 37 °C?	89.5 (65.5–98.2)	61.1 (43.5–76.4)	83.3 (71.0–91.3)	59.0 (42.2–74.0)
Food borne illness most frequent symptoms	100.0 (79.1–100.0)	77.8 (60.4–89.3)	95.0 (85.2–98.7)	71.8 (54.9–84.4)
Food borne illness agents transmission	73.7 (48.6–89.9)	52.8 (35.7–69.2)	65.0 (51.5–76.5)	56.4 (39.8–71.8)
Visual, olfactory or taste checks identify bacteria contaminated food?	42.1 (21.1–66.0)	41.7 (26.0–59.1)	45.0 (32.3–58.3)	51.3 (35.0–67.3)
How can MH contaminate meat?	73.7 (48.6–89.9)	55.6 (38.3–71.7)	85.0 (72.9–92.5)	56.4 (39.8–71.8)
MH can get ill in consequence of meat handling?	47.4 (25.2–70.5)	63.9 (46.2–78.7)	88.3 (76.8–94.8)	51.3 (35.0–67.3)
Health conditions that are not acceptable in food handling	47.4 (25.2–70.5)	36.1 (21.3–53.8)	65.0 (51.5–76.5)	30.8 (17.5–47.7)
Potential health consequences of animal intestinal bacteria	100.0 (79.1–100.0)	58.3 (40.9–74.0)	75.0 (61.9–84.9)	43.6 (28.2–60.2)
Listeria monocitogenes and food borne illness	68.4 (43.5–86.4)	36.1 (21.3–53.8)	53.3 (40.1–66.1)	33.3 (19.6–50.3)
Dirty and Clean workspaces in the abattoir	78.9 (53.9–93.0)	52.8 (35.7–69.2)	80.0 (67.3–88.8)	48.7 (32.7–65.0)
Food borne agents inactivation	52.6 (29.5–74.8)	50.0 (33.2–66.8)	56.7 (43.3–69.2)	48.7 (32.7–65.0)
Temperature of knives sterilisers	78.9 (53.9–93.0)	55.6 (38.3–71.7)	66.7 (53.2–78.0)	59.0 (42.2–74.0)

[a] Wilson procedure with a correction for continuity (Newcombe, 1998; Wilson, 1927).

ear and throat disease as conditions that are not acceptable in meat handling. Only 28.3% of BT ignored these conditions. Thirty four percent of the MH answered that only a skin disease is a non acceptable condition for meat handling. Sixty eight percent (67.5%) of the MH were aware of the need for skin injury protection in meat handling ($p = 0.009$, using Pearson Chi-Square test).

According to Jacob (1989), routine medical examinations of food handlers are of little value because they merely reveal the health status of the worker at a specific point in time. The author further states that these medical examinations are unreliable and that carriers of pathogens are unlikely to transmit these organisms. In this study, 72.4% of the respondents have indicated that they have been to routine medical examinations during the previous year, while 5.9% indicated that they have gone because they felt sick, whereas 12.5% needed to undergo medical examinations before employment. Food handlers must undergo medical examinations before employment to assess the general health. However, it has been suggested that routine medical examinations are regarded as not being cost-effective and, in fact, unreliable (Jacob, 1989; Nel et al., 2004).

3.2.1.5. Dirty and clean workspaces at the abattoir. Sixteen percent (15.8%) of GPFI, 44.4% of WSH, 20.0% of BT and 35.9% of NT have identified incorrectly all the dirty areas in the abattoir. Of all MH, 10% think that only the lairage is a dirty space, and 18% have only identified the room where offal are washed and prepared ($p = 0.001$, using Pearson Chi-Square test).

3.2.2. "Practice" questions (Table 4B)

3.2.2.1. Instruments and working surface cleaning. Eighty nine percent (88.5%) of the respondents were aware of the working surfaces and instruments washing and disinfection routine and correct steps and only 5.7% answered that they did not have contact with that operation. As far as disinfection is concerned, 25.3% of MH thought that sodium hypochlorite is the best disinfectant in meat industry but 47.4% were aware of the need for regular rotation of products for this purpose (Meyer, 2006). However, 12% did not know that, after the use of disinfectant on instruments and surfaces, both of them must be cleaned with potable water. Forty two percent (42.1%) of GPFI, 25.0% of WSH, 31.7% of BT and 30.8% of NT thought that non-potable water could be used for the cleaning of working surfaces and instruments. These differences were not statistically significant.

3.2.2.2. Personal hygiene. To the question "When do you wash your hands during a work day" only 3.2% of MH have not answered and 89.2% have answered that they washes them several times and whenever the activity is interrupted ($p = 0.181$, using Pearson Chi-Square test). To the question "different steps to correct hand wash", 5.8% of MH have not answered. The majority of MH referred all the steps for a correct hand wash, however 21.1% of GPFI, 38.9% of WSH, 30.0% of BT and 43.6% of NT have answered incorrectly, because they have not mentioned the use of nail brush ($p = 0.015$, using Pearson Chi-Square test).

Table 4B
Percentage of correct answers and 95% Confidence Intervals[a] (CI) of the questions "Practice" (qualitative results).

Questions "Practice"	% Correct Answers (95% CI)			
	GPF	WSH	BT	NT
	N = 36	*N* = 19	*N* = 60	*N* = 39
Working surfaces and instruments washing	84.2 (59.5–95.8)	94.4 (80.0–99.0)	90.0 (78.8–95.9)	89.7 (74.8–96.7)
Working surfaces and instruments disinfection products	47.4 (25.2–70.5)	36.1 (21.3–53.8)	58.3 (44.9–70.7)	43.6 (28.2–60.2)
Potable water use for washing purposes	57.9 (34.0–78.9)	58.3 (40.9–74.0)	61.7 (48.2–73.6)	59.0 (42.2–74.0)
Temperature ranges and meat preservation	36.8 (17.2–61.4)	27.8 (14.8–45.4)	38.3 (26.3–51.8)	25.6 (13.6–42.4)
Red Meat storage temperatures	100.0 (79.1–100.0)	63.9 (46.2–78.7)	83.3 (71.0–91.3)	74.4 (57.6–86.4)
Chopped meat storage temperatures	68.4 (43.5–86.4)	38.9 (23.6–56.5)	56.7 (43.3–69.2)	43.6 (28.2–60.2)
Freezing temperatures for meat	57,9 (34.0–78.9)	47,2 (30.8–64.3)	56,7 (43.3–69.2)	41,0 (26.0–57.8)
Different situations that imply hand washing before meat handling	100.0 (79.1–100.0)	100.0 (88.0–100.0)	86.7 (74.9–93.7)	84.6 (68.8–93.6)
Different steps to correct hand wash	78.9 (53.9–93.0)	52.8 (35.7–69.2)	70.0 (56.6–80.8)	43.6 (28.2–60.2)
Cross contamination and change of working instruments and clothes	21.0 (7.0–46.1)	27.8 (14.8–45.4)	31.7 (20.6–45.1)	33.3 (19.6–50.3)

[a] Wilson procedure with a correction for continuity (Newcombe, 1998; Wilson, 1927).

3.2.2.3. Temperature control. From the three ranges of temperatures presented, 0–4 °C/5–65 °C/70–80 °C, only 32.3% of the MH identified the range of 5–65 °C as the high-risk meat storing temperature. The GPFI group has also had the highest proportion of incorrect answers (63.2%), followed by BT (53.3%), WSH (52.8%) and NT (51.3%). Interestingly, the GPFI group seems to be confident regarding this topic since none of the respondents report "do not know" to this question, although the majority of the subjects has answered incorrectly. Seventy nine percent (78.6%) knew of the correct red meat storage temperature but only half of MH have reported the correct freezing temperature (50.6%) and the correct storage temperature for chopped meat (51.3%). If we consider professional training, WSH group has had a lower proportion of correct answers on red meat storage temperature (63.9%) than NT (74.4%). Twenty six percent (26.3%) of GPFI, 44.4% of WSH, 30.0% of BT and 23.1% of NT have answered incorrectly to the question about chopped meat storage temperature and 33.3% of NT answered "do not know" ($p = 0.036$, using Pearson Chi-Square test).

3.2.2.4. Change of clothes and instruments and cross contamination sources. Only twenty one percent (21.1%) of the GPFI, 27.8% of the WSH, 31.7% of BT and 33.3% of NT recognise the need to change clothes and knives by the end of the work at the abattoir (mainly in the first hours of the day), when they continue their tasks in the deboning room of the same building ($p = 0.087$, using Pearson Chi-Square test). Fifty seven percent (56.8%) of all MH recognise the need to change their protective clothing but do not admit the importance of replacing knives and 5.8% answered that is correct to carry their clothes and knives from the slaughter room into the deboning room. Regarding the porosity of surfaces, it can be observed that porous surfaces (clothes, aprons, sponges, etc.) show lower transfer rates when compared to non-porous surfaces as stainless steel and knobs (Kusumaningrum, Van Putten, Rombouts, & Beumer, 2002; Scott & Bloomfield, 1990). However, in this case, although apparently a lower risk might be associated to transfer from fabrics, it should be noted that the residual water (and eventually blood) accumulated in clothes would enable bacteria to survive for longer periods and, consequently, bacterial transfer events could also be prolonged (Bloomfield et al., 1994; Eustace et al., 2007; Rusin, Maxwell, & Gerba, 2002).

In addition to protective clothing fulfilling a safety function, 44.7% wear stainless steel mesh gloves. Stainless steel gloves also require cleaning and sterilisation, but these gloves are difficult to clean, due to their woven construction (Van Zyl, 1998). Upon asking the respondents about the frequency of cleaning, 59.5% have reported that they wash and sterilise their gloves several times a day, whenever they are visibly dirty (usually full of fatty or bloody deposits). Furthermore, a small percentage, 11.1% sterilises their gloves on a daily basis (end of work), while 22.2% have answered they never washed or sterilised their gloves because they were not connected with cleaning tasks. According to the Canadian Food Inspection Agency (CFIA), these gloves should be sterilised at regular intervals throughout the working shifts to prevent cross contamination between gloves and meat (CFIA, 1990; Nel et al., 2004).

On the matter of Pre-Requisite Plans (PRP) participation, 56.6% did not participate in any activity. The highest participation is related with cleaning activity, since 17.8% complete cleaning checklist forms and only 9.2% participate in meat temperature control activities, whereas 8.6% have maintenance related tasks.

4. Discussion

The questionnaire designed for the present study has allowed to detect quantitative differences in "knowledge" and "practice" skills among the participants. The satisfactory participation has permitted to highlight the existence of differences between MH who have and have not received professional training, obtaining the groups NT, WSH, GPFI and BT. This is remarkable and somewhat reassuring. Nevertheless, a further finer analysis of the content of the questions themselves (qualitative results) has not led to the same sense of reassurance. The proportion of correct answers in the MH groups who have had GPFI or BT training is significantly higher than the others from a statistical point of view, but results have also indicated that WSH training is not relevant to Food Hygiene and Food Safety knowledge and practice.

Regarding HACCP, which is a recent and relevant imposition of the EU Food Law, there was still a high proportion of MH (even with professional training, the WSH group) who were unacquainted with the concept. To the question "What is HACCP", only half of BT have answered correctly and this group has also had the highest proportion of incorrect answers, somehow contrary to what should be expected. It seems to be very difficult to implement an HACCP based system in this industry, when a high proportion of employees is not familiar with this reality and does not participate in PRP. Mortimore and Smith (1998) have shown that many trainers had been willing to provide HACCP training without considering the scope (what has to be taught and what need not) and the depth of coverage. Although numerous companies have developed, documented and implemented training programs, few understand why employee training is important, what their training requirements are, or how to assess the effectiveness of in-house training programs.

In the matter of meat storage temperatures, e.g. red meat, the WSH group has had the highest rate of incorrect answers and the lowest of correct answers. The BT group has not had better results, regarding the fact that they associate two different areas of professional training. A high proportion of GPFI, WSH and BT rely on visual, olfactory or taste checks to identify bacteria contaminated meat. This finding is difficult to explain, considering that they all have had professional training. The study demonstrates that there is also a general lack of knowledge on microbiological food hazards, i.e. *E. coli*, *Salmonella*, *Campylobacter*, *Yersinia* and *L. monocytogenes*.

It is generally accepted that the hands of food handlers are an important vehicle of food cross contamination and that improved personal hygiene and scrupulous hand washing lead to the basic control of spread of potentially pathogenic transient microorganisms (Allwood, Jenkins, Paulus, Johnson, & Hedberg, 2004; Daniels et al., 2002; Fry, Braden, Griffin, & Hughes, 2005; Lues & Van Tonder, 2007; Sneed, Strohbehn, Gilmore, & Mendonca, 2004). In this study, it has been possible to observe that in the four groups there are respondents who do not know all the steps for a correct hand wash. According to the results of Shojaei, Shooshtaripoor, and Amiri (2006), a dramatic reduction in hand contamination has been observed after a simple intervention that included a face-to-face health education on strict hand washing after visiting the toilet.

Concerning the topic of cross contamination, the majority of MH does not seem to be aware of the importance of changing clothes and working instruments, when they move from the tasks developed in "dirty spaces" (located at the abattoir) to "clean spaces" (deboning room). In addition, they also seem to have difficulties in identifying the differences between the spaces themselves. The UK surveillance system has reported that cross contamination was the main contributing factor (32%) for the outbreaks investigated in the period of 1999–2000 (WHO, 2003). Similarly, the US Centres for Disease Control and Prevention (CDC) have reported that 18 and 19% of food borne diseases caused by bacteria in the years 1993 and 1997 in the United States were associated with contaminated equipment and poor hygiene practices, respectively (CDC, 2000). Moreover, although most outbreaks result from extensive growth at abusive storage temperatures, insufficient cooking, etc., many are also associated with bacterial cross contamination/recontamination (Notermans,

Zwietering, & Mead, 1994; Roberts, 1990). Similarly, various authors have stated that cross contamination of bacterial and viral pathogens in homes and in food-service establishments could well be the major contributing factor to sporadic and epidemic food borne illnesses (Beumer & Kusumaningrum, 2003; Bloomfield, 2003; Chen, Jackson, Chea, & Schaffner, 2001). In the present study, a high proportion of respondents admits a potentially dangerous behaviour on a daily basis, as 56.8% ($n = 88$) recognise the need to change their clothes but do not admit the importance of changing knives when they end the work at the abattoir and start at the deboning room. In a HACCP based system perspective this is an unacceptable occurrence.

As a result of EU law implementation, Portuguese slaughterhouse and deboning room owners need to offer professional training to their employees but they do not show special concerns about their own training program and its contents. According to the evaluation of the present study, in a high proportion of MH who have had professional training in WSH, this training has not produced a significant contribution to meat safety. Furthermore, as several authors suggested, it seems that most managers in food and meat industry have a limited understanding of the global food safety strategy (Ehiri, Morris, & McEwen, 1997; Khandke & Mayes, 1998; Mortimore & Smith, 1998; Williams et al., 2003). MacAuslan (2003) has pointed out that the majority of food businesses do not have satisfactory training policies for all their staff. The author emphasized that too much reliance is being placed upon attaining a training certificate rather than attention paid to achieving competency in food hygiene practice. More emphasis and resources need to be diverted towards assisting managers to become highly motivated to food hygiene managers who develop and maintain a food safety background within their business. Few employers perceive a relationship between investment in their human resource assets and successful business performance, and training is often undertaken only to meet perceived statutory or inspection requirements (Pratten & Curtis, 2002; Seaman & Eves, 2006). Food business owners may be tempted to place the burden of training responsibility on an external employer, and not shoulder any responsibility themselves. This problem has two sides; firstly the employer lacks key management skills in leadership, motivation, training and evaluation, and secondly going for a certificate course as it is the "done thing" (MacAuslan, 2003). What we have observed in the present study is that the pressure to accomplish the law leads employers to get specialised training for their employees; however, there is no evidence that the worker practices improve when training programs provide only information (Nieto-Montenegro, Brown, & LaBorde, 2008; Rennie, 1994). Several studies have demonstrated that increasing knowledge does not necessarily lead to changes in behaviours (Clayton, Griffith, Price, & Peters, 2002; Ehiri et al., 1997; Rennie, 1994, 1995). To be effective, training programs should be based on appropriate adult education theory (Rhodes, 1988). In the present study, we have verified a low educational level of MH, the average formal education years being 6.5 (in Portugal the mandatory formal education takes 12 years) in a group with a mean age of 35 (Table 2), which may be a possible explanation factor for our results. The findings in the study of Toh and Birchenough (2000) affirmed education as an important link to the two variables: knowledge and attitudes; customs and environment. Some other authors suggest that the training programs should incorporate activities that support skills development relevant to real life situations in which the workers can put information into practice (Edmunds, Lowe, Murray, & Seymour, 1999; Kowalski & Vaught, 2002). Food hygiene training is a legal requirement within food industry and should be only one part of an effective food safety management strategy. Training will only lead to an improvement in food safety if the knowledge imparted leads to desired changes in behaviour at the workplace (Nieto-Montenegro et al., 2008; Seaman & Eves, 2006). To our knowledge, professional training of MH in Portugal has been "classroom based" and this study aims to contribute to a reflexion on the need for evaluation towards practical improvements.

Evidence from the literature suggests that food hygiene training as a mean of improving food safety standards is limited by a lack of understanding of those factors contributing to successful outcomes. Training activities closely associated with work environment would be more appropriate than food hygiene courses that operate divorced from the workplace and use solely knowledge-based assessment techniques (Seaman & Eves, 2006). The training of managers is a necessary precursor to the implementation of realistic food safety practices within the workplace. The effectiveness of training is very dependent on both management attitude and their willingness to provide the resources and systems for food handlers to implement good practices. There is a need to develop training methods that proved to change behaviour as well as imparting knowledge (Egan et al., 2007). Further research in issues including course content, training location, duration of courses, motivational factors and refreshment training is needed. Such research needs to be clearly thought out, well designed with good baseline data to achieve worthwhile results (Egan et al., 2007; Seaman & Eves, 2006). Seaman (2010) proposes the *Food Hygiene Training Model* which includes evaluation stages, managerial components and overall performance measures to take into account both the effective planning of the training program, the managerial support required to facilitate the training process, and the overall performance measures needed to ensure that training transfers into the required safe food handling behaviours. The proposed model incorporates three evaluation stages of the food handlers: 1) documented training needs with individual record, establishing a starting point; 2) knowledge test and/or practical skill assessment shortly after training, assessing any deficiencies in skills or knowledge at this stage; 3) food handlers evaluation of the training program to measure the perceived value and relevance of the training program, allowing respondents to portray approval or disapproval towards certain aspects of the training (Seaman, 2010). The overall performance measures include two final evaluation categories: the effect of food hygiene training on the individual food handler and the effect on the organization (Seaman, 2010). The success of training relies on the choice of the program, considering the relevance of the course to work activities, and providing food hygiene training in a language and at a level that allows the food handler to understand the content (Rennie, 1994; Seaman, 2010). Authors suggest that food hygiene courses should be shorter and focused on the needs and motivation of the participant, and include refresher training to provide both a physical and psychological environment conductive to food handler development and the enactment of safe food handling practices (MacAuslan, 2003; Rennie, 1994; Seaman, 2010; Worsfold, 2001).

The significance of the present results is limited in part by the sample size and by the fact that it has based on self-reported behaviour and practice. It is possible to conclude, however, that EU regulations have had a positive outcome in the matter of professional training of MH in Portugal. Operators, however, cannot rely on the fact that training has ever taken place. They must assume that all employees will need thorough, repeated training in the area of food hygiene and safety, as we observed that WSH training is not relevant to this aim (in spite of being relevant in terms of occupational safety and health). We suggest what can be a major concern in the moment of hiring new employees: to assess knowledge in food safety and promote immediate professional training, in addition to asking about previous work experience. In the present study, the MH show an average of 12.6 years of experience in the activity. However, the respondents have had poor results on the HACCP, microbiological hazards, temperature control, personal hygiene and cross contamination subjects.

In this activity, characterized by hard physical work and a traditionally low educational level of the workers, professional training should be adapted, with a strong connection knowledge-practice, considering motivational factors and beliefs. Behaviour changes in MH should be evaluated according to those conditions, encouraging the learning process and rewarding practical improvements.

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SECTION IV

GENERAL DISCUSSION

Salmonella has ascended, in the last decades, as the worldwide most important cause of foodborne outbreaks and one of the most significant bacterial agents causing human disease. In the EU, real efforts have been achieved in order to control this development. Particularly, in fowl populations, by the implementation of specific control measures, a consistent descendent trend was obtained. However, swine and pork persist as important reservoirs of MDR *Salmonella*.

The work presented in this PhD thesis answer several issues regarding the occurrence of MDR *Salmonella* in swine and pork, the cross-contamination process in the abattoir and the meat handlers level of knowledge and practice, concerning their participation in the overall problem. We present answers through the results below.

In this study one hundred healthy pigs presented to slaughter in eight abattoirs in the north of Portugal (districts of Braga and Porto) were sampled (ileocecal lymph nodes, carcasses and meat). In the respective deboning room, the hands of the meat handler responsible for the cut and deboning process of each sampled carcass were also sampled (Chapter 1, Section II). In each abattoir, it was sampled one pig from every different batch presented to slaughter. It was possible to identify 64 holdings from 11 districts from the continental territory of Portugal.

- **What is the occurrence of *Salmonella* in Portuguese slaughter swine, carcasses, meat and meat handlers?**

- **Is it possible to track *Salmonella* along different sampled material unveiling routes of cross-contamination?**

Salmonella was isolated in all abattoirs from both lymph nodes and carcasses, except for one abattoir where only one pig was sampled (Section III, Chapter 1). It was possible to observe a high *Salmonella* prevalence in slaughter swine (26%) and carcasses (16%), in accordance with previous Portuguese data (European Food Safety Authority, 2008; Vieira-Pinto et al., 2005). In the deboning rooms, *Salmonella* was isolated in 14% of meat samples and 9% of meat handlers' hands. Nine different *Salmonella enterica* serotypes were detected in the 60 positive samples: *S.* Typhimurium (53.3%; n=32) and the monophasic variant *S.* 4,[5],12:i:- (5%; n=3), *S.* Derby, (18.3%; n=11), *S.* Rissen (6.6%; n=4), *S.* Mbandaka (5%; n=3), *S.* London (5%; n=3), *S.* Give (3.3%; n=2), *S.* Enteritidis (1.6%; n=1) and *S.* Sandiego (1.6%; n=1). In order to track *Salmonella*

along diverse sampled material unveiling routes of cross-contamination, Pulsed Field Gel Electrophoresis (PFGE) following XbaI digestion of genomic DNA according to the standard 1-day protocol of the Centers for Disease Control and Prevention (CDC) (CDC, 2002) was undertaken. After PFGE analysis, isolates with a SD (Dice Band-based similarity coefficient) value > 85% were considered to belong to the same cluster and 17 PFGE profile types corresponding to 5 PFGE unique profiles (0006, 0008, 0011, 0014 and 0015) and to 12 clusters were identified (Section III, Chapter 2, Figure 1).

The results showed that even when the holding of origin of the pigs (Section III, Chapter 1, Table 3) and the collecting time periods were the same, different *Salmonella* clones were identified in the respective lymph nodes, suggesting a diverse *Salmonella* population in the previous environment (production level, transport and lairage). The prevalence at slaughter may be a result of cross contamination at the peri-harvest stage, including contamination from trucks, and also due to stress shedding induced by the trucking process and cross contamination during transport and holding (Dorr et al., 2009; Isaacson, Firkins, Weigel, Zuckermann, & DiPietro, 1999). On average, pigs stay in holding pens in abattoirs for about 3 h, which can be sufficient time for *Salmonella* contamination and establishment in the gastrointestinal tract and associated lymphatic tissues. Hurd and collaborators identified the lairage as a critical point of entry for *Salmonella* contamination, as some of the genotypic clusters identified in the lairage were subsequently obtained from the cecal contents or mesenteric lymph node samples (Hurd, McKean, Wesley, & Karriker, 2001). Another study conducted in pig barns showed that although the cleaning and disinfection protocol used effectively reduced the level of *Salmonella*, there was not complete elimination (Mannion, Leonard, Lynch, & Egan, 2007). This finding suggests that there is a need for multiple intervention procedures at different stages or perhaps more stringent disinfection protocols in swine production environments and holding pens in the abattoirs (Arguello et al., 2011; Mannion et al., 2007). Critical elements in the effectiveness of disinfectants have previously been shown to be even distribution on the surface, formation of biofilms, contact and drying times, and other factors related with the application protocol (McDonnell & Russell, 1999) and this should be integrated in the HACCP (hazard analysis critical control points) program of each abattoir. In spite of our work did not assess lairage contamination, it was possible to verify that most of the abattoir workers were not committed to cleaning tasks: only 17.8% completed cleaning checklist forms, and hygiene routines were widely different among abattoirs (Section III, Chapter 3).

Furthermore, in the study, we could trace the incidence of *Salmonella* along critical points to the slaughter process (evisceration, after preparation but before chilling and 24 hours later, in the deboning room). Disagreeing to what would be predictable,

abattoirs vertical scalding system, considered more hygienic, had higher carcass contamination results when compared with those abattoirs using the less clean system. This fact is possibly associated with other subsequent operations, as polishing and particularly evisceration, where are mostly relevant meat handler performance and good manufacturing practices (GMP) (Berends, Van Knapen, Snijders, & Mossel, 1997). This results also underlines the importance of meat handlers' training in work environment (Seaman & Eves, 2006) and internal verifying procedures in each abattoir, regarding good practice, for instance, not only in evisceration but also in equipment cleaning routines.

The number of pigs slaughtered per unit of time seems to be relevant to *Salmonella* occurrence in carcasses and meat. In the abattoirs studied, the ones with higher values (C and D) had worst contamination results (Section III, Chapter 1). This is probably related with a less cautious handling of pigs and carcasses as time is a constraint to correct developing of repeated tasks. In a number of consecutive carcasses from pigs which tested negative at the lymph node level, the same PFGE cluster was identified, suggesting that the origin of contamination was shared and possibly environmental (contact with equipment or meat handler) (Section III, Chaper 1). This pattern was mainly frequent in one abattoir (abattoir D), with 250 pigs slaughtered per hour. In abattoir H, a positive carcass was related to a positive lymph node sample from a previous animal, which contaminated contiguous carcasses in the slaughter line, disseminating the contamination to the cutting and deboning room, including the meat handler and several meat samples. In two abattoirs, in spite positive results from lymph node and carcass tests, there were no positive meat (B and G) or meat handlers (B) samples, most likely resulting from improved hygienic parameters and individual performance in the cutting and deboning rooms (Section III, Chapter 1). Nevertheless, in others (C and H) there was a causal link between the positive meat handler and the positive processed carcasses, emphasizing the importance of GMP, additional training, and the need of effective implementation of changes after training.

Several studies discussed an association between the slaughter of *Salmonella*-carrier pigs and carcass and meat contamination (Berends et al., 1998a; Botteldoorn, Heyndrickx, Rijpens, Grijspeerdt, & Herman, 2003; De Busser et al., 2011; Delhalle, Saegerman, Farnir, et al., 2009; Delhalle, Saegerman, Messens, et al., 2009; Vieira-Pinto et al., 2005; Vieira-Pinto et al., 2006). In our study, this association was confirmed in three positive pigs, as in each one the same PFGE clone was identified in the lymph node and subsequent samples (carcass and/ or meat). Indeed, this study identified high rates of *Salmonella* in both lymph nodes comparable to former studies (European Food Safety Authority, 2008; Vieira-Pinto et al., 2005) and in meat much higher than the most recent published Portuguese data (European Food Safety Authority, 2012a) but lower

than other author's report (Delhalle, Saegerman, Farnir, et al., 2009). In spite of there is causal relation among *Salmonella* contamination in carcasses and lymph nodes, this pathway seems to be less relevant when compared with other previous studies, underlining the repercussion of contamination spread through carcasses and meat and unveiling an important public health problem (Berends et al., 1998a; Botteldoorn et al., 2003; Botteldoorn et al., 2004; Vieira-Pinto et al., 2005; Vieira-Pinto et al., 2006). Cross-contamination among these different materials in the abattoir environment appears to have a greater influence than just the spread from positive lymph nodes. As other authors have formerly referred, the foremost contamination basis is probably an uninterrupted contamination cycle between slaughtered pigs, the environment, and the carcasses (Arguello et al., 2011; Botteldoorn et al., 2004; De Busser et al., 2011; Swanenburg, Urlings, et al., 2001).

In Portugal, the high percentage of *Salmonella*-positive (but normal in appearance) lymph nodes, has other public health implications, associated to Portuguese tradition of the consumption of pork bowel. The raw bowel (after the removal of lymph nodes) enters into the food chain through dispatch to butcher shops or meat plants where it is processed again, assuming a further role in *Salmonella* dissemination. The involvement of meat handlers in this process is possible as, in the performed survey, a high percentage of meat handlers identified incorrectly the dirty areas in the abattoir, namely the room where offals are washed and prepared (Section III, Chapter 3).

- **What is the meat handler's participation through the process of cross-contamination and *Salmonella* dispersion?**

In chapter 1 Section III, concerning hand contamination, 9% of meat handlers were positive to *Salmonella*. In three of them, the same PFGE cluster was the correspondent with that identified in previously manipulated carcasses and/or meat samples. This event recognizes a cross-contamination risk point and, again, a hygiene and public health problem, as in addition to the risk of infection, workers could act as a source for *Salmonella* transmission within the community. Hence, in Chapter 3 Section III of this thesis, we added and characterized meat handler's participation within the abattoir complex cycle of contamination, assessing their knowledge and practice.

It is recognized that of food handlers' hands are an essential link of food cross contamination and that improved personal hygiene and meticulous hand washing lead to the basic control of spread of potentially pathogenic agents (Allwood, Jenkins, Paulus, Johnson, & Hedberg, 2004; Daniels et al., 2002; Fry, Braden, Griffin, & Hughes, 2005; Lues & Van Tonder, 2007; Nel, Lues, Buys, & Venter, 2004; Sneed, Strohbehn, Gilmore,

& Mendonca, 2004). In the knowledge and practice survey that we performed, it was possible to verify that some of the meat handlers did not knew all the steps for a correct hand wash. Indeed, although a high percentage (88.5%) of meat handlers have claimed to be aware of washing and disinfection of equipment, only a small proportion participated in PRP (pre-requisite plans), namely by completing cleaning checklist forms. Moreover, it was possible to verify that Portuguese meat handlers' training it is still "classroom based" and detached from the workplace using solely knowledge-based assessment techniques, lacking valuation towards practical improvements. In addition, the survey demonstrated that half of the meat handlers did not seem to be conscious of the need of changing clothes and working instruments, namely knives, when they transfer from the tasks performed in 'dirty spaces' (located at the abattoir) to 'clean spaces' (deboning room) in the same meat plant. These knives, if previously used in evisceration, can easily constitute a vehicle of cross-contamination when used in the cutting and deboning room. In addition, they also seem to have difficulties in identifying the differences between the spaces themselves. It was also verified that respondent meat handlers, even after professional training, had a general lack of knowledge of microbiological food hazards, i.e. *Escherichia coli*, *Salmonella*, *Campylobacter* and *Listeria monocytogenes*, and the related risks to their own and public health. Moreover, approximately half of the respondents thought that they could identify whether meat is contaminated with food poisoning bacteria by visual, olfactory or taste checks and a high proportion believed that meat handlers can only contaminate meat if they are ill.

- **Is the serotype distribution the expected in swine and pork?**

The results concerning serotypes and their respective proportions are consistent with the Baseline EU survey and other studies of slaughtered pigs (De Busser et al., 2011; European Food Safety Authority, 2008; Vieira-Pinto et al., 2005), as *S.* Typhimurium is the most frequent in all groups of samples, followed by *S.* Derby, *S.* Rissen and *S.* 4,[5],12:i:- .The main variation was noticed in the lymph nodes, where all the serotypes were isolated except *S.* London, which was not previously reported in Portuguese lymph node data, but was recently identified in production (15.5%) and breeding-holdings (20%) (European Food Safety Authority, 2009). In carcass and meat samples only four serotypes were isolated, and *S.* Typhimurium and *S.* Derby were the most prevalent (Section III, Chapter 1, Table 2), which is compatible with the EU survey results on carcass contamination (European Food Safety Authority, 2008).

Importantly, the prevailing serotypes identified in this study are frequently associated with human disease (European Food Safety Authority, 2010a, 2012a; King, Lake, & Campbell, 2011), comprising the monophasic variant *S.* 4,[5],12:i:- (5%) regarded as emergent in humans (Dionisi et al., 2009; Hauser et al., 2010; Lucarelli et al., 2010; Switt et al., 2009) and *S.* Typhimurium DT104 (12%) (Antunes, Machado, & Peixe, 2006). The presence of this strain on a meat handler is a special burden, emphasizing the importance of the abattoir environment in the dispersion of human-health threatening *Salmonella* clones.

- **What are the resistance phenotypes, genotypes and genetic determinants in strains obtained from swine, carcasses, meat and meat handlers?**

Resistance was identified in 75% of all *Salmonella* isolates (58% lymph nodes; 75% meat handlers; 86% meat; 94% carcasses) being 63% MDR (resistant to ≥3 antibiotics structurally unrelated) (42% lymph nodes; 75% meat handlers; 79% meat; 81% carcasses) (Chapter 2, Section III). The results of our study, concerning lymph nodes, carcasses and particularly swine meat were much higher than others formerly described (European Food Safety Authority, 2008, 2012b).

Resistance to tetracycline (Tet) (n=42; 70%), streptomycin (S) (n=38; 63%) sulfamethoxazole (Su) (n=37; 62%), and amoxicilin (A) (n=34; 57%) was detected in a higher frequency than chloramphenicol (C) (n=9; 15%), trimethoprim (W) (n=5; 8%) and nalidixic acid (n=3; 5%), which may reflect the high usage of the first four antibiotics in food-producing animals (European Food Safety Authority, 2008). The most frequent resistance phenotypes were ASSuT (38%) ACSSuT (16%) SSuT (13%), agreeing with recent EU data (European Food Safety Authority, 2012b).The published evidence suggests that the growth-promoter ban in EU have reduced overall antibiotic use in animals (Guardabassi & Kruse, 2009; Phillips, 2007). However, some authors state that the use of growth promoters was accompanied by other, previously unrecognized, health promotional or prophylactic effects (Casewell, Friis, Marco, McMullin, & Phillips, 2003; Phillips et al., 2004; Phillips, 2007). After the removal of these antibiotics, animal welfare has suffered and regardless of the determinations to develop other aspects of husbandry, the veterinary use of therapeutic antibiotics, which are identical to those used in human medicine, has increased, and this constitutes a theoretical hazard to public health in relation to resistance in *Salmonella* (Casewell et al., 2003; Phillips et al., 2004). However, in spite of the recognition that some pathology increased after the complete termination of antimicrobial growth promoters (namely in Danish swine, there was a significant increase in antimicrobial treatments for diarrhea in the post-weaning period), other authors state

that there has been no major effect on productivity or feed efficiency in food producing animals (Arnold, Gassner, Giger, & Zwahlen, 2004; Laine et al., 2004; Wegener, 2003; Wierup, 2001a, 2001b). Additionally, authors state that resistant phenotypes may, however, persist at low but detectable frequencies for many years after removal of the selective pressures (Johnsen et al., 2009; Salyers & Amabile-Cuevas, 1997; Salyers, Gupta, & Wang, 2004; Schwarz et al., 2001).

- **Is it possible to identify in these sources MDR *Salmonella* clones with relevance in public health?**

S. Typhimurium clones (distributed by 9 PFGE types – T1-T9 – and including 2 unique profiles T6 and T9) were identified in swine and in meat handler's samples of 6 and 2 abattoirs, respectively (Section III, Chapter 2, Figure 1). Most clones included in five PFGE types (T1,T3, T4, T6, T7, T8); some clones were identified in diverse samples in the same abattoir as a result of cross-contamination, and in different abattoirs as a consequence of dissemination (Section III, Chapter 2, Figure 1). Mostly of them exposed structures (e.g. integrons and resistance genes) of the main human clones spread worldwide, including in Portugal (e.g. *S.* Typhimurium DT104) (Antunes et al., 2006). Particularly, T8 corresponding to *S.* Typhimurium DT104, isolated in lymph nodes, carcass, meat and a meat handler, presented a wide resistance phenotype (ACSSuT) as previously reported worldwide (Hur, Jawale, & Lee, 2012), diverse resistance genes [bla_{PSE-1}*, floR, aadA2, sul1, tet*(G)] and two class 1 integrons, respectively with 1000 and 1200 bp (base pairs).

The monophasic variant *S.* 4,[5],12:i:- isolated from swine in 3 abattoirs was clustered with *S.* Typhimurium (cluster IT), and mostly associated with ASSuIT type [bla_{TEM-1}*, strA-strB, sul2 and tet*(B)] (Section III, Chapter 2, Figure 1), typical of the European monophasic strains (Antunes et al., 2011; Dionisi et al., 2009; Hauser et al., 2010; Lucarelli et al., 2010; Soyer et al., 2009; Switt et al., 2009). Noteworthy, was here firstly reported the resistance phenotype ST, associated to *strA-strB, tet*(A) and *tet*(B) genes. This serotype has been considered emergent in EU and reveals high rates of antimicrobial resistance both in isolates from humans and pigs (European Food Safety Authority, 2012b).There was evidence that *S.* Rissen, also an emerging serotype (Hendriksen et al., 2008), had a clonal spread among swine and meat handlers of 3 and 1 abattoir, respectively (type R1). These clones presented different and wide resistance patterns [A(C)SSuTW], all including *tet*(A) genes encoding to tetracycline resistance, as also previously reported (Hendriksen et al., 2008). A specific class 1 integron (*dfrA12-orfF-aadA2*) as also observed as previously described in human and food products

(Antunes et al., 2006), which suggest the dissemination of these genes, by horizontal spread, between food-producing animals, industrial facilities, namely abattoirs and meat plants, and eventually consumers, with meat handler's participation. Eleven isolates of *S.* Derby, genetically related (type D1), were identified in swine from 4 abattoirs, and were associated with SSuT resistance phenotype, through the presence of *aadA2, sul1, tet*(A) encoding genes, all presented a class 1 integron (*aadA2*), also previously described in human and food products in Portugal (Antunes et al., 2006). *S.* London (type L1), recently identified in Portuguese swine (European Food Safety Authority, 2009, 2011a), presented the resistance phenotype ANSSuT and the genotype *bla*$_{TEM-1}$*, strA-strB, sul2* and *tet*(A), and was identified in both carcass and meat handler in two abattoirs. Clones of *S.* Mbandaka (types M1 and M2) presented resistance profile *strA-strB*, spread among three abattoirs. PFGE types T1, T3, T4, T6, T7, T8, D1, R1, and L1, which include 80% (n=48) of the isolates are mostly MDR (Section III, Capter2, Figure1). This study demonstrated clonal dissemination in different abattoirs and in diverse sampled materials, including meat handler's hands, suggesting the transference of strains between pigs, abattoir environment and humans and unveiling an important public health problem. In this process, abattoir operators assume a double risk position: they are agents of cross-contamination, but they also can get the infection and transport it into the community.

- **What is the level of general knowledge and practice in meat handlers from slaughter houses and meat plants?**

To our knowledge, this study performed the first professional training survey in Portuguese meat handlers, through a self-administered questionnaire assessing "knowledge" and "practice" (Section III, Chapter 3). Answers were obtained from all the meat plants contacted, but 10% of the employees have not returned the questionnaire. In the present study, we have verified a low educational level of meat handlers, the average formal education years being 6.5 (in Portugal the mandatory formal education takes 12 years) in a group with a mean age of 35 (varying widely between 16-58). An important proportion of the meat handlers (72.7%) had professional training in two diverse areas: Good Practice in Food Industry (12.03%) and Work Safety and Hygiene (22.8%); 37.9% of the respondents have had training in both areas. Nevertheless, 24.5% of the respondents have never had training. Meat handlers with professional training in Good Practice in Food Industry (GPFI) and in both areas (BT) have had the highest proportions of correct answers in Knowledge (66.92 ± 16.36 and 67.26 ± 21.05, respectively) and Practice questions (70.53 ± 17.47 and 68.67 ± 22.58). The proportion of correct answers

in the MH groups who have had GPFI or BT training is significantly higher than the others from a statistical point of view, but an additional analysis of the content of the questions themselves (qualitative results) have also shown that WSH training is not pertinent to Food Hygiene and Food Safety knowledge and practice (in spite of being pertinent in terms of occupational safety and health). Concerning HACCP, which is a current and pertinent requirement of the EU Food Law, there was still a high proportion of MH (even with professional training, the WSH group) who was unaware of the concept. To the question "What is HACCP," only half of BT had answered correctly and this group has also had the highest proportion of incorrect answers, conflicting to what should be predictable. It appears to be very problematic to implement an HACCP based system in this industry, when a high proportion of employees is not acquainted with this reality and does not participate in pre-requisite programs.

The results of this study point to the necessity to develop training, particularly in Good Practice in Food Industry, thus allowing meat handlers to improve their Knowledge and Practice. The implementation of evaluation criteria for the effectiveness of professional training is also critical as there is no evidence that the worker practices improve when training programs provide only information. What we have observed in the present study is that the pressure to accomplish the law leads employers to get specialized training for their employees. However, the success of training relies on the choice of the program, considering the relevance of the course to work activities, and providing food hygiene training in a language and at a level that allows the food handler to understand the content. Regarding the level of formal education observed within the respondent group, this limitation should be considered. There is also a need to develop training methods that proved to change behavior as well as imparting knowledge, considering that only 50.3% of MH with professional training believes that training provides useful information to their work. Furthermore, Food Industry operators cannot rely on the fact that training has ever taken place. They must assume that all employees will need detailed, repeated refreshment training in the area of food hygiene and safety, in spite of the previous work experience. In the present study, the meat handlers demonstrated an average of 12.6 years of experience in the activity. Nevertheless, the respondents have had poor results on the HACCP, microbiological hazards, temperature control, and personal hygiene and cross contamination subjects. Regarding the importance of the participation of meat handlers in the Food Chain and considering that 64.9% admitted their interest in future training the improvement opportunity should not be neglected.

SECTION V

CONCLUSIONS AND PERSPECTIVES

This work describes evidence founded on original studies of the occurrence and characterization of MDR *Salmonella* isolates in slaughter swine products and meat handlers, and on the first Portuguese meat handlers' knowledge and practice survey. Based on these primary results, it seems appropriated, as final conclusions and perspectives, to refer:

Our data suggest that swine and pork meat are a significant source of MDR *Salmonella* and that abattoir processes could endorse its contamination. While swine could harbor *Salmonella* previously to slaughter, the abattoir environment can contribute to further cross-contamination along the slaughter line, including contact with meat handlers. In order to improve standards in the post-harvest pork meat chain, measures have to be taken at these stages. Reducing the prevalence of *Salmonella* positive pigs at the primary production phase can significantly decrease one of the main sources of contamination at the abattoir. However, strong evidence is clear: hygiene of slaughter operations, and meat handlers' practice should also be improved. Guidelines and procedures to reduce *Salmonella* are an essential requisite in abattoirs and the warranty of its accomplishment is critical. Moreover, they should highlight the requirement for safe food-handling practices in abattoirs and meat plants to reduce the level of *Salmonella* occurrence in carcasses and pork, emphasizing the role of the meat handlers' effective training and the control of the implementation of an improved practice. Meat handlers' training, as it has been generally developed in the abattoir and meat plants' context, it is not being an efficient tool to acquire a robust knowledge and to adopt a safe practice. As previously referred, in this activity, characterized by hard physical work and a traditionally low educational level of the workers, professional training should be adapted, with a strong connection knowledge-practice, considering motivational factors and beliefs. Behavior changes should be evaluated according to those conditions, encouraging the learning process and rewarding practical improvements, namely towards a safe handling goal. Additionally, GMP and current HACCP implementation remain crucial to reduce cross-contamination and to maintain the level of *Salmonella* burden as low as possible in abattoir environment and pork meat, protecting public health. Cooperation between Veterinary Authorities and abattoir and meat plants' Operators, is required in order to achieve effective advances in meat safety.

The identification of MDR *Salmonella* clones in swine, pork and meat handlers, as well as the appearance of emergent international clones, namely *S.* Typhimurium DT104, the monophasic variant *S.* 4,[5],12:i:- and *S.* Rissen with wide resistance patterns, is of

great concern. This fact recognizes an insufficiently studied transmission path, relevant in public health, and requires an intervention. Furthermore, the circulation of animals and food products all over the EU multiplies the possibilities of acquisition of new strains and intensifies this MDR threat. The surveillance of antimicrobial resistance to follow the emergence and spread of MDR *Salmonella* from these sources seems critical. Particularly, *S.* 4,[5],12:i:-, here primarily reported with a new resistance phenotype, and assuming an increasing relevance worldwide, has been scarcely characterized and requires a close surveillance at a national level. *S.* London, recently identified in an EU base line survey in Portuguese swine, to our knowledge, firstly reported in this work with a wide MDR pattern, requires to be followed. Our results, revealing high frequency of MDR phenotypes to veterinary most used antibiotics, support the need of a revision of preventive measures, infection-control strategies and interventions in primary production (e.g. biosecurity, vaccination, good surveillance, rapid detection and treatment).

The development of cooperation programs involving Veterinary Authorities and veterinarians in swine herds is essential. These programs should include monitoring antimicrobial susceptibility and MDR among *Salmonella* spp. isolated from environment and clinically ill animals. This assessment should comprise molecular characterization with structural and functional data, which are essential for understanding the emergence of new resistance mechanisms and virulence, and to control its spread into the food chain and the community.

SECTION VI

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